Neuronal ICAM-5 Regulates Synaptic Maturation and Microglia Functions

Sonja Delalu (Née Paetau)

Molecular and Integrative Biosciences
University of Helsinki

and

Doctoral School in Health Sciences
Doctoral Program Brain & Mind

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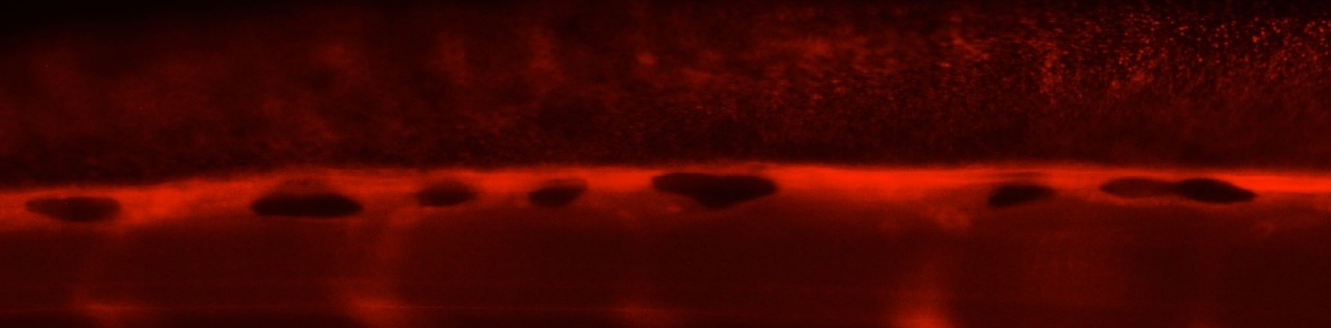

Supervisor	Professor Carl G. Gahmberg University of Helsinki
Thesis Committee	Docent Sari Lauri University of Helsinki Docent Petri Ala-Laurila University of Helsinki Docent Mikaela Grönholm University of Helsinki
Pre-examiners	Docent Pirta Hotulainen Minerva Foundation Institute for Medical Research, Helsinki Docent Michael Courtney University of Turku
Opponent	Professor Tarja Malm University of Eastern Finland
Custos	Professor Kari Keinänen University of Helsinki

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”…och så vandrade de vidare och med dem vandrade stigen.”

Tove Jansson
Kometen kommer, 1946

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ORIGINAL PUBLICATIONS

The thesis is based on the following articles, which are referred to by their Roman numerals (I-IV) in the text. In addition, some unpublished data is included. Publication I is reprinted by permission from Springer Nature: Springer Cell adhesion molecules: implications in neurological diseases by P.S. Walmod and V. Berezin, Springer Science+Business Media New York 2014. Publication III is reprinted by permission from Springer Nature: Springer Encyclopaedia of signalling molecules by S. Choi. Springer International Publishing AG 2017.

I. **ICAM-5 – a neuronal dendritic adhesion molecule involved in immune and neuronal functions**, Carl G. Gahmberg, Lin Ning and Sonja Paetau. Cell adhesion molecules: implications in neurological diseases P.S. Walmod and V. Berezin ads. Springer. 2014;8:117-32. Doi: https://doi.org/10.1007/978-1-4614-8090-7_6

II. **ICAM-5 affects spine maturation by regulation of NMDA receptors binding to α-actinin**, Lin Ning, Sonja Paetau, Henrietta Nyman-Huttunen, Li Tian and Carl G. Gahmberg, Biology Open, 2015 Jan 8;4(2):125-36. Doi: 10.1242/bio.201410439

III. **Intercellular adhesion molecule-5**, Sonja Paetau and Carl G. Gahmberg, Encyclopaedia of signalling molecules, 2nd Ed, Springer, Editor S. Choi. 2017 9 Dec. Doi: https://doi.org/10.1007/978-3-319-67199-4_101656

IV. **ICAM-5 inhibits microglia adhesion, phagocytosis and promotes an anti-inflammatory response in LPS stimulated microglia**, Sonja Paetau, Taisia Rolova, Lin Ning and Carl G. Gahmberg. Frontiers in Molecular Neuroscience, 2017 22 Dec. 10:431. Doi: 10.3389/fnmol.2017.00431

ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
AMPA	2-Amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
APLP	Amyloid precursor like protein
ARF	ADP-ribosylation factor
ATP	Adenosine triphosphate
CAM	Cell adhesion molecule
BDNF	Brain-derived neurotrophic factor
CaMKII	Ca/Calmodulin dependent kinase 2
CD	Cluster of differentiation
CNS	Central nervous system
CR	Complement receptor
CSF	Colony stimulating factor
Del-1	Developmental endothelial locus-1
DIV	Day *in vitro*
ECM	Extracellular matrix
ERM	Ezrin/radixin/moesin
FACS	Fluorescent activated cell sorting
GABA	Gamma amino butyric acid
GFAP	Glial fibrillary protein
GDNF	Glia-derived neurotrophic factor
GTP	Guanine triphosphate
HBSS	Hank's balanced salt solution
IBA	Ionized calcium binding adaptor molecule
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IgSF	Immunoglobulin superfamily
IL	Interleukin
INF	Interferon
kDa	Kilo Dalton
KO	Knock out
LFA-1	Leucocyte function-associated antigen-1
LPS	Lipopolysaccharide
LTD	Long-term depression
LTP	Long-term potential
Mac1	Macrophage-1 antigen
MIDAS	Metal ion dependent adhesion site
MMP	Matrix metalloproteinase
NCAM	Neuronal cell adhesion molecule
NMDA	N-Methyl-D-aspartate
PBS	Phosphate buffered saline

PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PSD	Postsynaptic density
RIL	Reversion-induced LIM protein
SNARE	SNAP (soluble NSF attachment protein) receptor
SynCAM	Synaptic cell adhesion molecule
TGF	Transforming growth factor
TLCN	Telencephalin
TNF	Tumour necrosis factor
VLA	Very late antigen
WT	Wild type

ABSTRACT

The human brain continues to fascinate generation after generation of neuroscientists. Our knowledge is expanding at an accelerating rate, yet the details of memory formation and information processing remain an enigma. The purpose of this work has been to provide novel insights into how the brain operates on a molecular level, with the focus on one particular protein, the intercellular adhesion molecule (ICAM)-5.

The human brain is more than just the sum of its components. It is fundamental that the different cell types that reside in the brain work together in perfect harmony, each playing their own role, still in tune with each other. In this dissertation I have investigated the ICAM-5 mediated communication between neurons and the resident immune cells of the brain, the microglia. First, we identified a molecular mechanism, by which ICAM-5 plays a role as a negative regulator of spine maturation. In the young spine, ICAM-5 competes with glutamate receptors for binding to the cytoskeletal anchor α-actinin. Synaptic transmission induces a cleavage of the extracellular ICAM-5, and the maturation process of the spine is allowed to proceed. Next, we showed that the consequentially solubilized fragment of ICAM-5 is bound by microglia and affects them. Soluble ICAM-5 inhibits phagocytosis and promotes an anti-inflammatory phenotype in immune challenged microglia.

Taken together, these results suggest that ICAM-5 is a versatile molecule that plays a role in synaptic maturation and immunology. It is tempting to speculate on a role for ICAM-5 in synaptic pruning, however this line of research remains in the future scope for now.

1. It takes more than neurons to make a brain

Our brain is what really makes us human. The central nervous system, CNS, of the human body is a fascinating machinery coding everything in the spectrum ranging from a single muscle twitch to our individual personality. The network of neurons together with a scaffold of glia cells offers functions still not replicable in synthetic devices.

The neurons form the basis of information processing and storing. There are approximately 10^{11} neurons in the adult brain that form an astronomical amount of connections and potential information routes. The general architecture of the brain forms during development, but fine details of the networks are under constant maintenance and reconstruction. The contact sites between neurons, the synapses, form the computational element and these structures have the capability to reshape in a process called plasticity. It is this astonishing remodelling of the brain that is the key to learning processes, such as rehabilitation after brain injury (Nudo, 2013).

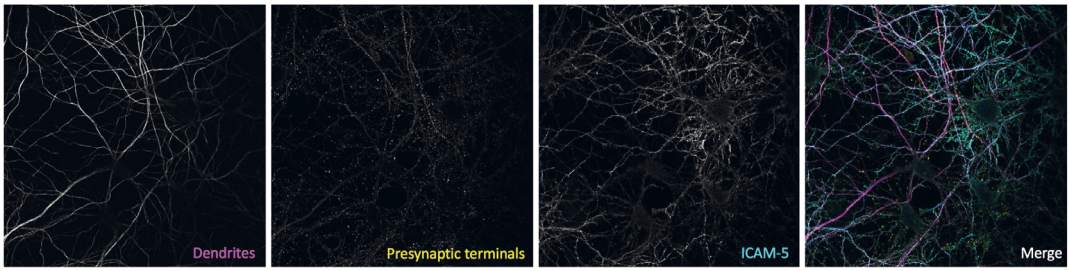

Figure 1. Hippocampal neuron cultures from mouse show an elaborated network of neurites and synaptic contacts after 14 days in culture. Dendrites are sprinkled with presynaptic terminals that correspond to points were two neurons communicate. (Purple: Microtubule-associated protein-2, yellow: synapsin-1)

Calculated in numbers, glia cells comprise about 90% of the cells in the human brain (Greter and Merad, 2013). There are several types of glia cells perfectly adapted to a specific task. Non-neural cell types include astrocytes, oligodendrocytes, ependymal cells and microglia. Interestingly, astrocytes and microglia are in close contact with synapses and regulate several aspects of synaptic function. Astrocytes influence brain plasticity by supporting synapses, recycling neurotransmitters and monitoring the state of the synapse. They wrap around the synapse and can even engulf it if necessary (Theodosis et al., 2008). Microglia are the most dynamic and active cells of the brain, scanning the brain parenchyma and keeping the garden of synapses tidy. They originated from leukocyte precursors, and in case

of an immunological challenge, they form the first line of defence. In the healthy brain however, they keep the environment clean from cellular debris (Wake et al., 2013).

In addition to neurons and glia cells the CNS is also inhabited by the cell types associated with vascularisation, such as endothelial cells. Except for microglia, there are also casually infiltrating lymphocytes, especially T cells. During normal conditions the brain is rarely visited by lymphocytes.

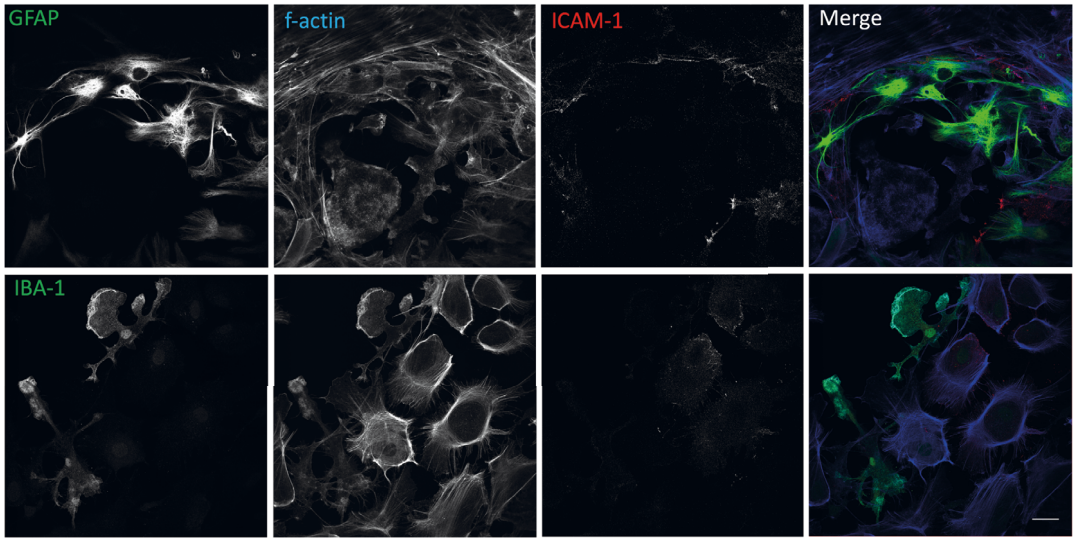

Figure 2. Glia cells isolated from mouse brain. GFAP (glial fibrillary protein) is a marker for astrocytes, while ionized calcium binding adaptor molecule (IBA)-1 is a marker for microglia. The cell cultures are triple stained for the cell marker in green (first column), filamentous actin in blue (second column) and the cell adhesion molecule intercellular adhesion molecule (ICAM)-1 in red (third column). In the column to the right, all separate channels have been merged. ICAM-1 is only weakly expressed in normal glia calls and it is absent from microglia. Scale bar: 25 µm.

2. Synaptic maturation and plasticity enables learning and memory

The brain has the essential ability to reshape upon experience. The process is regulated through a molecular interplay that is mainly triggered by synaptic transmission. For some modalities in mammals, the plasticity of the brain is constrained to certain time windows. Such a critical period is prominent in the development of vision. During early postnatal development, the signalling pathways through the optical chiasm to the primary visual cortex are formed. If these contacts are not properly established during this critical time frame it can hardly be reversed (Morishita and Hensch, 2008). Critical windows illustrate the fact that the general layout of the network is present at birth, but refinement requires activity-dependent maturation. On the cellular scale the construction and plasticity of neuronal circuits it is at its peak activity during early postnatal development. A large number of excess synapses are formed, which are later trimmed. The actively transmitting contacts

compete out the weaker inputs. Synaptic plasticity can be categorized as either short-term or long-term in mammals (Penn, 2001). Long-term plasticity described above persist from minutes to years and modulates the transmission efficacy of an existing synapses and networks. It can lead to the formation of new synapses or the removal of existing ones. Short-term plasticity, on the other hand, is more subtle and describes changes to an existing synapse (Fioravante and Regehr, 2011; Zenke and Gerstner, 2017).

2.1 Dendritic spines are dynamic structures

Along the dendritic shafts small protrusions emerge, mature and disappear during the course of development and plasticity. They first appear as thin filopodia that mature into thin, stubby, and finally mushroom spines, figure 4. The role of these structures is to offer a contact platform for axons and to form the postsynaptic components of novel excitatory synapses. During the maturation process, there is a prominent change in protein composition and turnover of actin filaments in the spine. Actin is the cytoskeletal component of the spine and it can be bundled in parallel fibres or branched. Actin dynamics play an essential regulatory role in spine formation and morphogenesis (Ethell and Pasquale, 2005; Halpain, 2000; Hotulainen and Hoogenraad, 2010; Sekino et al., 2007). The filaments in filopodia and young spines undergo active threadmilling where the filaments are depolymerized at the slow-growing end and synthesized at the fast-growing end. The polymerization process requires adenosine triphosphate (ATP) and is in spine development regulated by small guanidine triphosphate (GTP) phosphatases. The Rho family of GTP phosphatases, including Rho, Rac and Cdc42, are at a key position here (Scott et al., 2003).

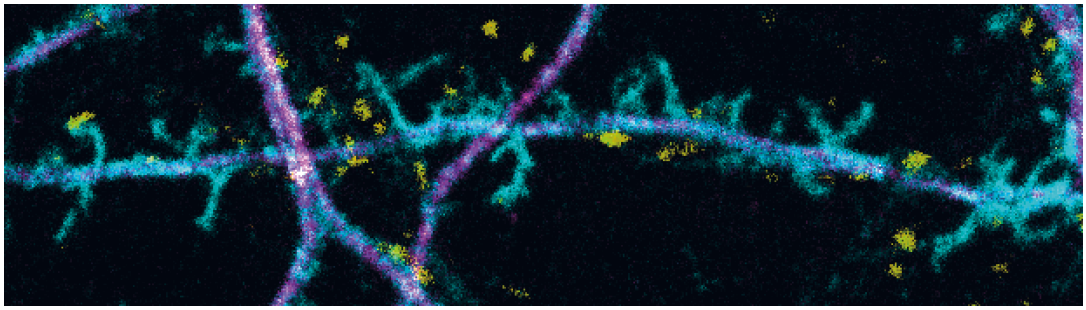

Figure 3. Filopodia are small protruding structures from a neuronal dendrite, here seen in a hippocampal neuronal culture that has been cultured for 14 days *in vitro*. Filopodia are the precursors of mature spines and ICAM-5 (cyan) is highly enriched in them. Presynaptic elements are seen as yellow dots (synapsin-1) and a dendritic marker is seen in magenta (microtubule-associated protein-2).

Filopodia are the precursors of spines and they typically contain actin networks from base to the shaft. The actin can be both branched and linear of variable length (Korobova and Svitkina, 2010; Li et al., 2016). As the filopodia start to enlarge at the tip, the actin filaments become branched to form the spine head (Hotulainen and Hoogenraad, 2010, Halpain, 2000). Filopodia can be visualized by an immunofluorescent staining for ICAM-5, as shown in figure 3.

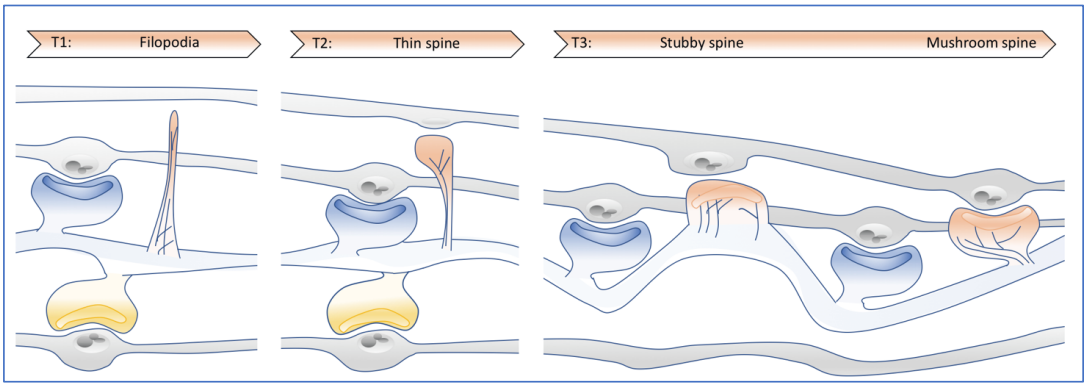

Figure 4. Filopodia are dynamic structures. Dendritic spines can be stable (blue structure), unstable and disappear (yellow structure) after they have gone through a maturation process ranging from filopodia, to thin spine, to mature stubby or mushroom spine (brown structures). Spines can adopt various shapes and much of the shift can be explained through actin dynamics. However, the maturation process might not be as linear as has been previously thought. T1-T3: time point 1-3. Axons are in grey and the central dendrite is in light blue. Actin filaments are shown in the maturing spine as blue lines.

Spines develop a branched actin filament matrix that is stabilized when the spine is mature. For example, myosin IIb has been shown to have the ability to stabilize the actin filaments (Sekino et al., 2007). When the spine head is in contact with a presynaptic terminal or an axonal bouton, glutamate receptors and postsynaptic density (PSD) concentrate in spine heads, anchored to the actin cytoskeleton (Yoshihara et al., 2009). The molecular architecture of a mature mushroom spine, as well as in the presynaptic terminal, is highly organized (Perez de Arce et al., 2015).

Equally important to spine maturation and stabilization is the elimination of exuberant spines. During normal development, an excess of synaptic structures is formed. They are then refined and only the correctly wired synapses remain. If a spine fails to make a successful synaptic connection, which is activity-dependent, it is destined for elimination (Riccomagno and Kolodkin, 2015). Spine elimination is less studied as compared to spine formation, however, collapse of the actin cytoskeleton is known to be one mechanism. Long-term depression (LTD) induces cofilin-dependent spine shrinkage, *in vivo* (Zhou et al., 2004). During dendritic pruning, the process where excess contacts are removed, the dendrite undergoes apoptosis-like events. First, the cytoskeleton is destabilized, then the

structure is fragmented and eventually the debris is cleaned away (Williams et al., 2006). Spine dynamics are represented in figure 4.

2.2 The synapse: information processing in a marriage of four

The excitatory synapse is an asymmetric engagement of the axonal presynaptic terminal connected to the postsynaptic structure. The neuronal elements are further wrapped by astrocytic membrane and the synaptic cleft is frequently visited by microglial protrusions, figure 5. Adhesion molecules play a crucial role in the initial formation of a synapse and the eventual stabilization of it, if the transmission is sufficient.

On the presynaptic terminal, the active zone assembles and vesicles with neurotransmitter accumulate. The hallmark scaffolding proteins include bassoon and piccolo and voltage gated calcium channels are the functionally most important channels in signal propagation and vesicle fusion. On the postsynaptic terminal, glutamate receptors accumulate in the middle of the contact site and adhesion molecules are organized to the peripheries (Perez de Arce et al., 2015).

The synapse is in many aspects an optimized structure for information processing (Adrian et al., 2014). The presynaptic terminal contains primed vesicles with neurotransmitters that are docked to the membrane through SNAP (soluble NSF attachment protein) receptor (SNARE) proteins. This readily releasable pool of vesicles is docked in an orderly fashion, close to the voltage gated calcium channels. The dynamics of neurotransmitter release is regulated based on the activity of the neuron. Mainly the intracellular calcium concentration and the number of docked vesicles and their regeneration can either up- or down regulate the amount of released neurotransmitter, respectively (Fioravante and Regehr, 2011). The postsynaptic terminal is in a similar way well suited for information integration. The shape of the spine isolates the input to a small volume and allows for specific modifications, depending on the intensity of the transmission. The prime molecular mechanism of experience dependent learning at the level of a single synapse is long-term potentiation (LTP), with the counteracting LTD (Bliss and Collingridge, 1993).

LTP is a mechanism regulating the efficiency of the excitatory synapses, resulting in a general stabilization of active synapses and enhanced synaptic efficacy (Kumar, 2011). When glutamate is initially released, it activates 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) receptors. AMPA receptors are ligand gated cation channels and the opening of them causes a depolarization of the postsynaptic membrane. These channels are mainly permeable to sodium and potassium. Simultaneous transmission in a closely neighbouring synapse or a burst of repeated firing in one synapse leads to a situation where the postsynaptic membrane is already depolarized when the subsequent action potential and release of glutamate takes place. In this case, the depolarization of the membrane has released the N-methyl-D-aspartate (NMDA) receptors of their inhibitory magnesium block and the NMDA receptors can conduct an ion flux. NMDA receptors are similar to AMPA receptors, only they are also permeable to calcium. The entry of calcium initiates signalling

that is the basis for early LTP (Kumar, 2011). A collective series of events leads to the accumulation of AMPA receptors in the postsynaptic membrane and hence, the following release of glutamate will have an increased effect.

Early LTP described above is independent of protein synthesis, while continuous signalling of that same synapse leads to protein synthesis and the construction of novel dendritic architectures in the late LTP (Lee et al., 2005). Late phase LTP is the result of protein synthesis and one such protein that is important for synaptic maturation is brain-derived neurotrophic factor (BDNF). It is mainly expressed in neurons and cleaved into a mature form by proteases like furin in the Golgi apparatus or by matrix metalloproteinases (MMPs) or plasmin in the extracellular matrix (Pang et al., 2004; Panja and Bramham, 2014). The mature form signals through the tropomyosin-related kinase receptor B, and promotes neuronal survival and maturation, while the pro-form signals through p75 receptors, promoting apoptosis (Teng, 2005). BDNF can also be produced by microglia and induce spine maturation (Parkhurst et al., 2013).

Many of the downstream effects seen in LTP are conveyed by the action of Ca/Calmodulin dependent kinase 2, CaMKII. It is an abundant, multi-isomeric holoenzyme of 12 subunits that is activated by a rise in the free intracellular calcium concentration through the action of calmodulin. Calcium-bound calmodulin binding to CaMKII induces an auto phosphorylation of the enzyme. The phosphorylated form of the protein is active due to the rejection of a regulatory segment and it can target several downstream effectors. The intracellular calcium concentration is tightly regulated and decreases rapidly after a rise. The phosphorylation of CaMKII is on the other hand more long-lasting and can hence be regarded as a molecular form of memory (Lisman et al., 2012). The rapid phosphorylation of the critical threonine 286 and the slow decay of activity in CaMKII further allows it to integrate subsequent calcium spikes on a physiologically relevant timeframe (6-8 seconds). Thus, CaMKII is important for the initiation of spine plasticity, however less so for the maintenance of the plasticity (Chang et al., 2017).

The remodelling of exciting spines, and the emergence of novel ones, like in late LTP, requires modifications of the extracellular matrix (ECM). Extracellular endopeptidases like MMPs are truly important in synaptic plasticity (Huntley, 2012). Not only do they degrade the ECM to allow for spines to grow, they also cleave adhesion molecules. Many adhesion molecules can be truncated by endopeptidases and they can exist as full-length, integrated in the membrane, or as soluble fractions of the extracellular domain. As soluble proteins, they have different signalling capabilities as compared to the membrane bound form. Proteases indicated in plasticity include soluble MMPs and membrane bound disintegrin and metalloproteinase (ADAMs) and they target cell adhesion molecules (CAMs) such as the neuronal cell adhesion molecule (NCAM), synaptic cell adhesion molecule (SynCAM), L1CAM and ICAM-5 (Conant et al., 2015).

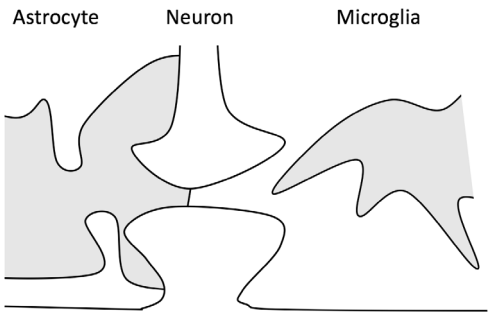

Figure 5. The quadripartite synapse consists of the pre- and postsynaptic terminals, enveloped by the astrocytic membrane, and visiting microglia protrusions.

In the model of the quadripartite synapse, the neuronal elements are isolated by a perisynaptic astrocytic shell. Astrocytes play an important role in removing and recycling neurotransmitters and in modulating the function of the synapse. Astrocytes take up glutamate through the glial high-affinity glutamate transporter and the glutamate transporter-1 (Perego et al., 2000) and buffer the perisynaptic potassium concentration through Na^+/K^+ ATPase. In addition to potassium and neurotransmitters, they maintain the homeostasis of the pH, reactive oxygen species and calcium. To support the energy metabolism of the synaptic elements, astrocytes provide them with lactate as a source of energy (Ghézali et al., 2016). Synaptic activity is mirrored in astrocytes as calcium waves and like neurons, astrocytes have a debated capacity of releasing vesicular content based on the existence of SNARE proteins and small, electron dense vesicles (Bohmbach et al., 2018). Astrocytes further play an important role in synapse maturation and refinement. During synaptic maturation, astrocytes secrete glypican-4. It induces signalling in the presynaptic terminal and subsequent release of neuronal pentraxin-1. Through this molecular mechanism, postsynaptic AMPA receptors accumulate and the synapse is strengthened (Farhy-Tselnicker et al., 2017). Astrocytic input is required for the refinement of cortical wiring on a synaptic scale. During synaptogenesis, it is common that cortical dendrites are multi-innervated. Intracortical and thalamocortical innervation competes for contact and through pruning, one excitatory connection remains. Astrocytic hevin has been shown to modulate this competition in the favour of thalamic input (Risher et al., 2014). Hevin is an ECM molecule localized to the synaptic cleft.

In conclusion, the concept of the quadripartite synapse defines the communication between pre- and postsynaptic elements, perisynaptic astrocytic and microglia protrusions as the fundamental property of information processing. While the glial sheath covers most of the CNS synapses, the microglia protrusions are more dynamic and transient. In this perfectly tuned quartet, they play the same melody in individual scales that make synaptic plasticity harmonic.

2.4. α-actinin as a master puppeteer of glutamate receptors in synaptic plasticity

Glutamate, released in excitatory synapses activate four types of ionotropic glutamate receptors, classified by structural homology and their sensitivity to synthetic AMPA, NMDA or kainic acid. The functional cation channel is tetrameric, composed of four subunits,

strictly originating from the same molecular family. The AMPA receptor family has 4 subunits (GluA1-4), the NMDA receptor family has 7 subunits (GluN1, GluN2A-D and GluN3A-B) and the kainate receptor has 5 subunits (GluK1-5). In addition to these, there are two delta receptors, GluD1-2, that make the ionotropic glutamate receptor family comprising of 18 members that has been characterized thus far. Each subunit consists of an amino terminal domain, a ligand-binding domain, a transmembrane domain and an intracellular carboxyl terminal domain. The transmembrane domain consists of three membrane-spanning helices and one membrane re-entrant loop (Traynelis et al., 2010).

The cytoplasmic tail is important for receptor mobility and efficacy and often regulated by phosphorylation. Mainly the extracellular part, but also the intracellular tail of many glutamate receptors act as substrates for a variety of proteolytic enzymes. Cleavage of the cytoplasmic tail leads to receptor degradation and reduces synaptic efficacy, while truncation of the extracellular domain can potentiate the receptor. One such case is plasmin mediated proteolysis of the amino-terminal domain of GluN2A that removes its inhibitory zinc binding site (Yuan et al., 2009).

AMPA receptors exert the basal glutamatergic transmission, NMDA receptors are vital coincidence detectors in synaptic plasticity, while kainate receptors regulate excitability both pre- and post-synaptically. Due to the lack of endogenous agonists, the function of delta receptors has remained enigmatic. GluD1 was first implicated in high frequency hearing, while mice devoid of GluD2 showed impaired synaptic function in Purkinje cells. Accumulating evidence suggests that GluD receptors have functions beyond channel activity, such as metabotropic endocytosis of AMPA receptors in LTD (Contractor et al., 2011).

NMDA receptors are typically formed by two homodimers and GluN1 is an obligatory subunit (Traynelis et al., 2010). However, a functional NMDA receptor can also be formed by a GluN1 homodimer combined with a GluN2 heterodimer. These subunit compositions have distinct expression patterns in the CNS and the various combinations generate receptors with different functions. GluN1 and GluN3 bind glycine, while GluN2 binds glutamate. The GluN1/GluN3 receptor is hence activated by glycine alone, although these receptors might not form under physiological conditions. GluN3 subunits are most likely expressed as tri-heteromers and function as such as response-limiting receptors, since they have a reduced conductance and they downregulate GluN1/GluN2 receptor trafficking (Das et al., 1998).

As mentioned above, the postsynaptic terminal is highly organized and rich is actin (Li et al., 2016; Perez de Arce et al., 2015). Linked to the actin cytoskeleton, scaffolding proteins such as PSD-95 have their designated localizations. Membrane integral proteins are anchored to the cytoskeleton through these scaffolding proteins and this binding is mainly regulated through phosphorylation. α-Actinin is one such synaptic protein that couples actin with adhesion molecules and channels and it is hence an important mediator of synaptic maturation. α-Actinin constitutes a family of four, mostly calcium sensitive actin-binding

proteins within the spectrin superfamily. It forms an anti-parallel dimer with a central rod domain, consisting of four spectrin repeats. The spectrin repeats are flanked by an actin binding domain on one side and a calcium sensitive calmodulin-like domain on the opposing side. The calmodulin-like domain contains four EF-hand motifs that can bind calcium in the cytoskeletal isoforms (1 and 4) of α-actinin. The binding of calcium induces a conformational change that impairs the actin crosslinking capacity of α-actinin. Muscular isoforms of α-actinin (2 and 3) connects actin to the Z-disk complex. (Drmota Prebil et al., 2016). The spectrin repeats form a binding platform for various proteins, including CAMs and glutamate receptors (Liem, 2016).

α-Actinin is known to interact with both metabotropic and ionotropic glutamate receptor and it is through these, implicated as an underlying mechanism for filopodia formation and synaptic maturation. Due to technical limitations (mainly the lack of good antibodies), many studies do not separate between the different family members. However, some specific functions have been identified. α-Actinin-1 interacts with type-1 metabotropic glutamate receptors and induces the formation of filopodia. Additionally, α-actinin-1 binds non-phosphorylated GluA4, that might be linked to synaptic targeting by an protein kinase A (PKA) mediated, activity-dependent mechanism (Nuriya et al., 2005). In the absence of α-actinin-2, filopodia accumulate and they fail to mature into mushroom shaped spines (Hodges et al., 2014), indicating that α-actinin-2 promotes spine maturation. α-Actinin-4 binding to CaMKII was identified as a mechanism by which excitatory metabotropic glutamate receptor signalling induces spine head enlargement (Kalinowska et al., 2015). AMPA receptors were also suggested to be imported into spines through an α-actinin dependent process where a linking protein, reversion-induced LIM protein (RIL), binds α-actinin through one domain and AMPA receptors through another (Schulz et al., 2004).

In the rat striatum, α-actinin-2 was found to interact with both GluN1 and GluN2B subunits. Immunoprecipitations of glutamate subunits or α-actinin-2 showed only moderate associations, indicating that only a minority of the NMDA receptors are bound by α-actinin-2 (Dunah et al., 2000). The membrane proximal intracellular region of GluN1 can be bound by calmodulin, CaMKII and α-actinin. In this region of GluN1, calmodulin can be accompanied by either α-actinin or CaMKII. Calcium influx through NMDA receptors activates calmodulin that in turn dislodges α-actinin from GluN1 and stabilizes CaMKII binding instead. Displacing α-actinin from GluN1 inactivated the NMDA receptor (Merrill et al., 2007).

α-Actinin can further modulate GluN2B function through the interaction with CaMKII. In a calcium independent manner, α-actinin can bind CaMKII and stabilize its interaction to the subunit GluN2B containing glutamate receptors. This interaction promotes the CaMKII mediated phosphorylation of the serine 1303 in the GluN2B subunit, and simultaneously inhibits the phosphorylation of the serine 831 in the GluA1 subunit (Jalan-Sakrikar et al., 2012).

In addition to NMDA receptors binding to calmodulin and α-actinin, NMDA receptors also associate with membrane-linked phosphatidylinositol-4,5-bisphosphate (PIP_2) through α-actinin. This was suggested to facilitate NMDA receptor activation by promoting a fully activated conformation of the NMDA receptor in a model by Michailidis et al. (Michailidis et al., 2007). PLCγ mediated degradation of PIP_2 releases α-actinin and the associated NMDA receptor cytoplasmic tails from the cell membrane. Several G-protein coupled membrane receptors, such as the nerve growth factor that signals through the receptor tyrosine kinase A, can activate phospholipase C (PLC). Activated PLC catalyses the hydrolysis of PIP_2.

Taken together, NMDA receptors are regulated at many levels and through various mechanisms. α-Actinin is in a key position, linking GluN1 and GluN2B to various intracellular proteins that conveys regulatory actions on the receptor. It is clear that this complicated network of mechanisms must act spatially and temporally distinct from each other.

3. Microglia: immune cells and beyond

The brain is well isolated from the rest of the body. It is enclosed by three layers of meninges and is encapsulated by the skull. Due to its high demand for energy, it has an elaborate network of blood vessels, but these are tightly controlled by astrocytes that enwrap them and selectively pick up nutrients. The brain has its own immune system, consisting mainly of only one cell type, microglia, figure 6. Microglia infiltrate the brain at a very early developmental stage and become resident. They are long-lived cells with very limited, if any, refill from the peripheral leukocytes later in life. Some studies have shown that tissue macrophages are able to infiltrate the brain, in an age dependent manner (Greter and Merad, 2013). Microglia have their origin in the primary haematopoiesis from myeloid precursors in the yolk sac and can regenerate from local progenitor pools in the brain. Unlike macrophages, microglia develop independently from the cytokine colony stimulating factor (CSF)-1, even though possessing the CSF-1 receptor (Ginhoux et al., 2010; Greter and Merad, 2013). Since they were discovered by Rio-Hortega a century ago, they have mainly been studied in the light of neuroinflammation. As they are phagocytosing cells by profession and belong to the innate branch of the immune system, they form the first and probably most important line of defence and they clear away cellular debris under non-pathological conditions. Undoubtedly, they are the main source of proinflammatory cytokines in neurodegenerative diseases and brain injury and they are important in the resolving phase of injury, cleaning away cellular debris (Krause and Müller, 2010). However, a growing body of evidence has identified these cells in an emerging role in synaptic pruning and plasticity. It has become evident, that these cells are not dormant cells, springing to life only to cause destruction. Ground-breaking *in vivo* imaging work convincingly showed that microglia are highly motile and dynamic cells under physiological conditions (Davalos et al., 2005; Nimmerjahn et al., 2005). Since then, huge effort has been put into unravelling the mystery of what these cells are up to. The dynamic fine protrusions can slip inside of the synaptic cleft and one microglia cell can contact several synapses at the same time (Tremblay

et al., 2010). It is now clear that they are much more than mere immune cells, and an exciting example of how a peripheral system, previously thought to be dormant in the brain, has been adopted to serve another function. Microglia come equipped with the complete machinery for phagocytosis and their talent has been put to use in the removal of excess synapses, guided by complement components. The hallmark studies of microglia-neuron interactions and their main findings since 2005 are represented in Table 1.

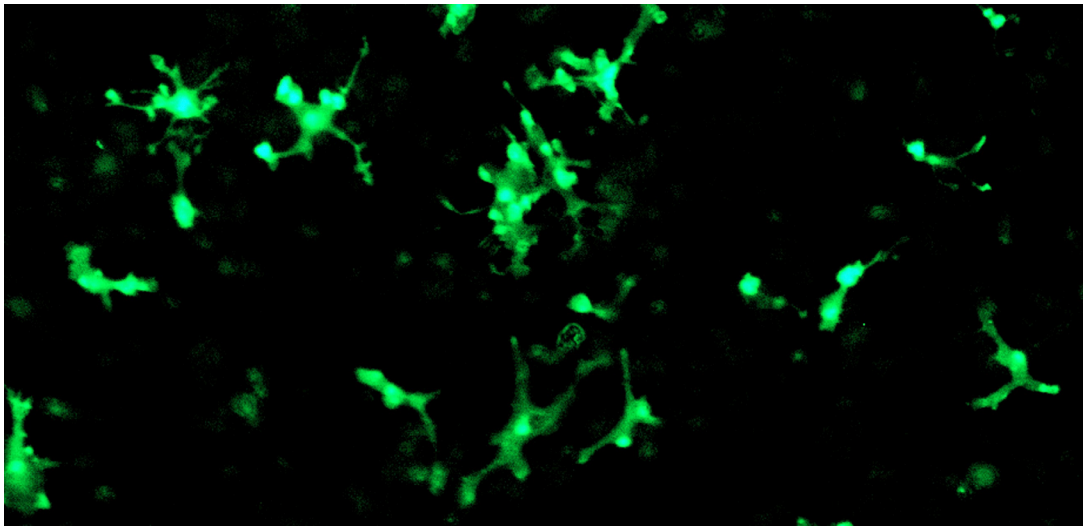

Figure 6. Microglia in culture. The cells are very dynamic and mobile. Even in culture they retract and extend their processes, constantly patrolling their environment.

3.2. Predators or scavengers? A feast for microglia with complements

The complement cascade is an innate mechanism, targeting pathogens for destruction and phagocytosis by professional eating cells, such as macrophages. Complement activation can be initiated by several mechanisms, all leading to the collection of a cascade of components in an orderly fashion on the surface of the bacteria. In the classical pathway, the C1q complex assembles on immunoglobulins bound to the surface of the pathogen. The alternative pathway relies on the C3 component that is spontaneously cleaved to C3a and C3b. C3b can directly bind pathogens and induce phagocytosis through the complement receptors 3 (CR3, magrophage-1 antigen (Mac-1), integrin αM/β2, CD11a/CD18) (Sarma and Ward, 2011).

Mice deprived of the complement components, C1q, C3 or CR3 showed an impairment of synaptic pruning and synaptic development. This raised the question of the role of microglia in this process, since they are the resident cells expressing CR3 in the brain (Stevens et al., 2007). Microglia processes were seen to contact dendritic spines and presynaptic elements in the healthy brain (Wake et al., 2009), further suggesting that microglia might be involved in the maintenance of the neuronal networks.

When microglia were first studied *in vivo*, microglia processes were seen to be attracted to a site of injury and by extracellular ATP (Davalos et al., 2005). Following studies focused on microglia in the intact brain and the dynamics and targets of the moving microglia extensions. Processes were seen to scan (extending and retracting in an exploratory fashion) the brain parenchyma, spending a few minutes in direct contact with synaptic elements. Ischemic terminals obtained increased microglial attention, and the terminals were later lost (Wake et al., 2009). The dynamics of microglia processes has since been studied in various settings and the results have been somewhat contradictory. As a rule, it seems that microglia are under tight spatial and temporal regulation.

Evidence for synaptic elements being phagocytosed by microglia first came from the studies in the visual system, where neuronal membranes were found in lysosomes in microglia (Tremblay et al., 2010). It was concluded that microglia are actively pruning during CNS development in mice (Paolicelli et al., 2011). Catching microglia in action, however, proved to be a more challenging task. Studies on microglia interaction with spines, indicate that sensory experience function as a regulator of microglia activity (Tremblay et al., 2010). The normal visual stimuli compared to light deprivation or subsequent light stimuli gave rise to microglia associated with the corresponding synapses in different modalities. During light deprivation, microglia showed an altered morphology. The motility of microglia was reduced and they concentrated at large existing spines, causing them to shrink in a similar way as in LTD. The shrinking spines were recovered when the rodents were re-exposed to light.

The hunt for molecular mechanism that drive and direct the pruning process was set in motion. Two signalling pathways were targeted. The first chemokine to be investigated was fractalkine (CX3CL1), which is mainly expressed by neurons and its unique receptor (CX3CR1), is only expressed by microglia in the brain. Fractalkine can be expressed as a membrane bound adhesion molecule, or cleaved from the membrane surface as a soluble chemokine. Genetic modification of the receptor showed that microglia motility was reduced if fractalkine signalling was disrupted (Liang et al., 2009). Even though it has been suggested that fractalkine receptor signalling was responsible for a reduced pruning rate (Paolicelli et al., 2011), recent studies has however ruled out a role for fractalkine signalling in microglia pruning, at least in the male visual system (Lowery et al., 2017; Schecter et al., 2017). It seems like the previously observed neuronal abnormalities in the fractalkine receptor impaired mouse line is more due to other impairments in microglia, such as reduced motility, rather than directly pruning.

Secondly, the complement cascade was suggested and proved to be directing microglia in the sculpting of neuronal architecture. Evidence first came from a CR3 knock out mouse line, where pruning was impaired at postnatal day 5 (P5) and synaptic density remained abnormal at P35 (Stevens et al., 2007). An elegant study (Schafer et al., 2012) on the role of microglia in synapse remodelling in the postnatal retinogeniculate system shed light on the pruning ability of microglia. The process is neuronal activity- and complement-receptor dependent (Stephan et al., 2012). In the synapses of retinogeniculate neurons, complement cascade components are expressed. These components, such as C3, are important for the

synaptic maturation and pruning. The microglia specific phagocytic pathway involves recognition of C3 in the presynaptic terminal by the C3 receptor Mac-1, which was indeed identified as a regulator of the pruning process (Schafer et al., 2012). Microglia tend to prune synapses of the weaker circuit, even though the molecular mechanism by which microglia decide between active and inactive synapses remained unresolved. Soon after, transforming growth factor- β (TGF-β) from astrocytes was shown to instruct neurons in producing the complement component C1q that tag synapses for elimination (Bialas and Stevens, 2013).

Main finding	Reference	
Microglia are highly motile cells that extend their processes towards an injured site through an ATP mediated process	Davalos et. al.	Nat Neurosci. 2005
Microglia are highly motile and constantly scan the brain parenchyma. BBB disruption rapidly attract microglia that shield the injured site	Nimmerjahn et. al	Science. 2005
Immature astrocytes induce neurons to express C1q, which localizes to synapses. C1q and C3 are necessary for normal synaptic pruning	Stevens et al.	Cell 2007
Microglia contact synapses in an activity-dependent manner and ischemic terminals receive prolonged inspection, followed by the loss of the synapse.	Wake et al.	J Neurosci. 2009
Microglia engulf synaptic material and fractalkine signalling is partially included in this process	Paolicelli et al.	Science. 2011
TGF-β regulates neuronal expression of C1q in synaptic pruning	Allison et. al.	Nat. Neurosci. 2013
Microglia secrete BDNF to promote a learning-dependent synapse formation	Parkhurst et al.	Cell 2013

Table 1. Since the ground-breaking *in vivo* imaging of microglia in 2005, much effort has been put into the research of microglia-synapse interactions. The key studies and their main findings are presented here (Bialas and Stevens, 2013; Davalos et al., 2005; Nimmerjahn et al., 2005; Paolicelli et al., 2011; Parkhurst et al., 2013; Stevens et al., 2007; Wake et al., 2009).

3.3. The yin and yang of inflammation on a neural chessboard

The invite that neurons express inflammation-related molecules, and microglia express molecules and receptors related to neuronal transmission, invites us to believe that the cells in the brain are more intertwined in function than previously believed. Neurons direct the function of microglia, and the outcome can vary on a spectrum ranging from neuroprotective to neurotoxic (Biber et al., 2007).

Cytokines and chemokines are the soluble carriers of inflammation throughout the body. Their expression and signalling in the brain is associated with both physiological processes and inflammatory events. Proinflammatory cytokines have long been implicated in age-dependent cognitive decline (Labrousse et al., 2012), although recent genome wide expression studied showed that immune-related genes were upregulated under normal memory retrieval and extinction events (Scholz et al., 2016). TGF and tumour necrosis factor

(TNF) genes were associated with memory retrieval, while interleukins (ILs) were associated with memory extinction.

In the context of memory formation, IL-1 has been vastly studied. It is considered a proinflammatory cytokine, since it is upregulated by lipopolysaccharide (LPS) stimulation (Bilbo et al., 2008). IL-1 is expressed as IL-1α or IL-1β and both forms bind the type 1 interleukin receptor (IL-1R1) and IL-1ra is an antagonist for IL-1R1. Evidence exist showing that IL-1 has both a detrimental and a beneficial effect on hippocampal-related memory formation and maintenance (Goshen et al., 2007). Under pathological conditions IL-1β expression in the CA1 region can be negative for memory formation, while in young mice, IL-β signalling is required for the same experimental task (Takemiya et al., 2017). This example illustrates the complexity of the cytokine balance and the accuracy required in spatial and temporal expression.

Although all cells of the CNS are capable of producing cytokines, microglia are the main source of soluble factors related to immunology. Microglia have been suggested to be activated through two pathways, the classical or the alternative (Aguzzi et al., 2013). In the classical pathway the activating cytokines are interferon (INF)-γ or LPS, while cytokines of the alternative pathway are IL-4 and IL-13 (Olah et al., 2011). The alternative activation pathway is thought to bring microglia to a state where their function is neuroprotective since microglia in this case release neurotrophins, the cytokine portfolio is not neurotoxic and phagocytosis is controlled. Neuroprotective microglia function in the reuptake of excess glutamate, removal of cellular debris, and production of insulin-like growth factor (IGF)-1, glia-derived neurotrophic factor (GDNF) and BDNF. Healthy neurons may secrete factors that reduce microglia activation, such as TGF-β, BDNF and fractalkine (Cardona et al., 2006). The cytokine profiles of microglia that are related to an anti-inflammatory or a proinflammatory response is listed in table 2.

Proinflammatory	Anti-inflammatory
IL-1β	IL-1ra
IL- 2	IL-4
IL-6	IL-10
IL-6	IL-13
IL-12	TGF-β
IL-15	CCL13
IL-17	CCL4
IL-23	CCL17
IFN-γ	CCL18
TNF-α	CCL22
CCL2	CCL23
CCL3	CCL24
CCL4	CCL26
CCL5	BDNF
CCL8	IGF-1
CCL11	GDNF
CCL12	
CCL15	
CCL19	
CCL20	
CXCL1	
CXCL1	
CXCL9	
CXCL10	
CXCL11	
CXCL13	

Table 2. Microglia are the resident immune cells of the brain and they secrete soluble factors that can be proinflammatory or anti-inflammatory. Interleukin (IL), interferon (IFN), Tumour necrosis factor (TNF), Chemokine (C-C motif) ligand (CCL), Chemokine (C-X-C motif) ligand (CXCL) transforming growth factor (TGF), Brain-derived neurotrophic factor (BDNF), Insulin-like growth factor (IGF).

Probably the most intensively studied agent in the field of inflammation is TNF-α. Several successful clinical applications target TNF-α signalling and can alleviate the symptoms in diseases such as rheumatoid arthritis (Feldmann, 2002). LPS stimulation induces a robust TNF-α expression in microglia, an effect that could be dampened by neuronal fractalkine hence reducing the neurotoxic effect (Zujovic et al., 2000). This cytokine is however not only associated with destructive inflammation. TNF-α is required in synaptic scaling after

sustained reduction in activity and it likely comes from microglia. Synaptic scaling is required for a synapse to perform optimally even under sustained reduction or increase in activity. As opposed to LTP and LTD that takes place on the level of individual synapses, scaling entails all synapses of a neuron. The main mechanisms include the modulation of glutamate receptor subunit composition and their abundancy (Beattie et al., 2002).

Secreted factors convey communication between neurons and microglia, not only in immunological events or in synaptic plasticity, but also in neuronal injury and regeneration. ATP released by damaged neurons, can activate the metabotropic P2Y or inotropic P2X receptors in microglia, which activate the cells. The activation of the P2Y12 receptor by ATP activates the β1 integrins, which are essential for the extension of the microglial processes towards the lesion. β1 integrins bind to collagen in the extracellular matrix and are considered to be involved in the polarized protrusions in the activated microglia (Ohsawa et al., 2010). The activation of the β1 integrins has also been shown to induce proliferation in microglia. Another chemotactic clue released by damaged neuronal tissue is the chemokine CCL21, which stimulates the receptor CXCR3 in microglia, promoting microglia migration (Rappert et al., 2002).

Microglia have been suggested to contribute to neurogenesis (the birth of new neurons from progenitor cells) in the sub ventricular zone if the microglia have been activated by factors released from apoptotic or ischemic cells. In this case, microglia up-regulate the expression of TGF-β and thus promotes acceleration of progenitor proliferation (Olah et al., 2011). Fractalkine signalling has also been implicated in hippocampal neurogenesis. Mice devoid of the fractalkine receptor had lower rates of hippocampal neuronal regeneration, in addition to deficiencies in motor learning and cognitive functions (Rogers et al., 2011). The same effect was seen in exercise induced neurogenesis in the hippocampus. When fractalkine was neutralized by antibodies, the formation of neurospheres was reduced in mice that had been allowed to run (Vukovic et al., 2012).

In conclusion, microglia continue to surprise and amaze by their staggering versatility. The more we learn, the more complex and harder it becomes to explain the interplay between different cell types in the brain. It is clear, that it certainly takes more than neurons to make a brain and that all aspects of cell communication need to be taken into consideration when studying learning and memory, as well as pathological events.

4. Neuronal adhesion molecules in synaptogenesis and plasticity

Cell adhesion is of outmost importance in development, cell signalling and migration. Adhesion is promoted through specific membrane integrated proteins that are able to bind elements of the ECM or ligands expressed on the membrane of other cells. The CAMs are often anchored to the cytoskeleton, they are highly dynamic and spatially regulated on the membrane surface. In synaptogenesis and synaptic plasticity, the interplay between homo- and heterophilic adhesion molecules provides target recognition, contact stabilization, pre- and post- synaptic specializations and activity-dependent plasticity (Missaire and Hindges,

2015). Adhesion molecules share several structural moieties that allows them to be divided into classes. The repetition of similar structures, such as the immunoglobulin (Ig) fold and the fibronectin (FN)-III repeat gives families of protein variability and flexibility. In the intracellular domain, many synaptic CAMs have a PDZ-domain-binding motif, that give them direct contact to scaffolding proteins such as the PSD-95 family that contains three PDZ domains. Common classes of CAMs in the CNS can be divided into the following groups: 1. Immunoglobulin superfamily, IgSF, 2. cadherins, 3. integrins, 4. neurexins and 5. neurolignin. Ephrins form an additional important family of adhesion associated molecules that are implicated in synaptic maturation and plasticity. The EphB/EphrinB pair has been implicated in filopodia formation by their interaction with actin modulating small GTP phosphatases.

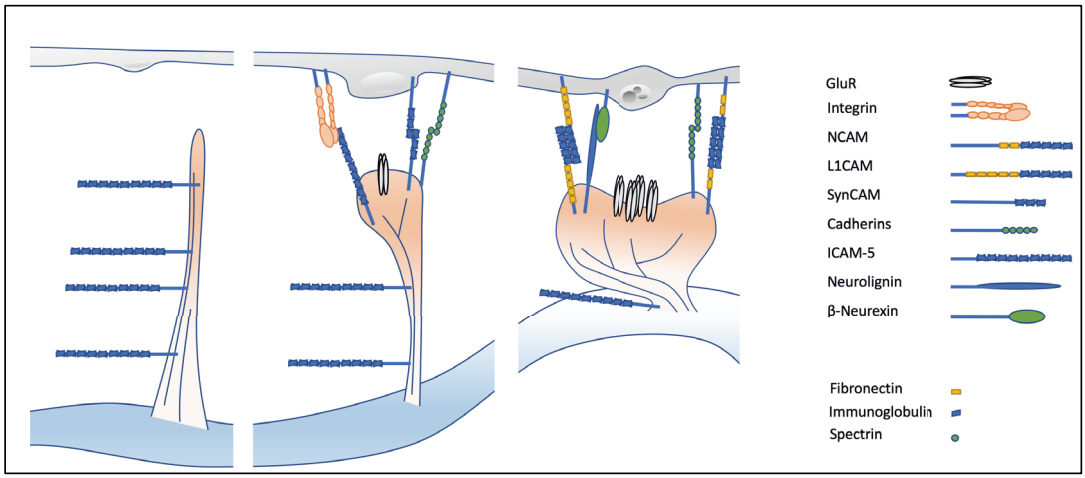

Figure 7. Adhesion molecules are involved in synaptogenesis and they can promote maturation through stabilization of the synaptic structures. ICAM-5 induces the formation of filopodia. ICAM-5-integrin binding, as well as homophilic cadherin and SynCAM binding are important in the initial contact formation between the dendrite and the axon. The stable synapse is characterized by an organized pattern of trans-synaptic adhesion on the rim of the contact area, while glutamate receptors accumulate at the centre.

IgSF adhesion molecules are the most common types of adhesion molecules in the brain. A double-layered β sheet sandwich fold characterizes the Ig domain. The β sheet consists of four common strands in the antiparallel β sheet structure (Bork et al., 1994).

NCAM holds an important position in neuronal adhesion. It is involved in the generation and stabilization of neuronal structures such as neuronal migration, axonal outgrowth and synapse formation. NCAM has the ability to form homophilic binding between two molecules, as seen in synapses (Dityatev et al., 2004). Structurally, the main isoforms of NCAM typically have two fibronectin (FN)III, and five Ig domains in the extracellular part. The Ig domains are situated distal to the FNIII domains (Zhang et al., 2008b). NCAM can be modified by the addition of polysialic acid. This modification renders the molecule anti-

adhesive and is hence a powerful regulatory switch. Functionally, NCAM can contribute to both pre- and postsynaptic specialization by the intracellular interaction with spectrins. In the cortex, NCAM regulates intrinsic excitability of pyramidal cells and the inhibitory tone that they receive (Zhang et al., 2017).

Another crucial, neuron associated member of the IgSF is the L1CAM. In addition to the six Ig domains, L1CAM also contains five FNIII repeats. It is capable of homophilic binding by the Ig domains, in addition to other ligands, such as integrins. L1CAM is mostly implicated in early neuronal development, where it induces neurite sprouting through dephosphorylation and hence activation of cofilin (Figge et al., 2012; Zhang et al., 2008b).

The 4-membered family of synaptic CAMs (SynCAM) is heavily implicated in excitatory synapse formation in vertebrates (Biederer et al., 2002). The SynCAMs have three extracellular Ig domains and bind in homophilic trans across the synaptic cleft. Presynaptic SynCAM signalling through CASK can influence the expression of voltage gated calcium channels and hence contribute to the site-specific specialization. Accompanied by glutamate signalling, SynCAM was able to form functional synapses independently (Biederer et al., 2002). Albeit their fairly simple structure, SynCAMs have proven to be prime drivers of excitatory synapse assembly.

The second class of abundant adhesion molecules in the CNS is the cadherins. All cadherins have the characteristic approximately 100 amino acid cadherin repeats in the extracellular domain and are calcium sensitive. These glycoproteins bind elements of the ECM as well as other cadherins by homophilic binding. This homophilic binding is seen in synapses as a stabilizing factor where the binding is characterized by the cadherins being in a stronger adhesive cis-state (Togashi et al., 2002). N-cadherin is the hallmark cadherin in the CNS and it is recruited to the synapse by synaptic activity (Yam et al., 2013). It is considered a late phase stabilizing adhesion molecule that is involved in late phase maturation, and less so in spine formation. Cadherins can influence synaptic specialization through catenins (Togashi et al., 2002).

In addition to the classical families of synaptic CAMs, the amyloid precursor protein (APP) family has recently gained an increasing body of evidence in their role in synaptogenesis. Mammals express three members in this family, APP and amyloid precursor like proteins (APLP)-1 and -2. Structurally, the APPs are type-1 integral proteins and share two heparin-binding domains and one zinc-binding domain in the extracellular part. APP and APLP-2 have a functionally important copper-binding domain that can reduce Cu(II) to Cu(I). All proteins in this family localizes to pre-and postsynaptic elements and are linked to synaptic signalling molecules. Their expression is upregulated during early postnatal life, at the time of active synaptogenesis. Hence they show typical characteristics of other CAMs (Cousins et al., 2015; Schilling et al., 2017).

4.1. The curious integrin family is everywhere and gossiping inside and out

Integrins are widely expressed adhesion molecules in the animal kingdom. They are heterodimeric type I membrane spanning proteins consisting of a non-covalent combination of one α chain and one β chain. There are 17 different α chains and 8 β chains. The integrins have the ability to mediate both inside-out and outside-in signalling in the host cell. Ligand-binding promotes outside-in signalling as it induces an intracellular signalling cascade. Inside-out signalling, on the other hand, is driven by phosphorylation of the intracellular tails of both α and β chains, thus modulating the affinity for the ligand. Both types of signalling are conveyed through the conformation of the heterodimer. β2 and β7 integrins are leukocyte specific (Gahmberg et al., 2009).

The integrins are structurally complex membrane proteins that serve as receptors for the ECM, cellular adhesion ligands and other molecules. The I-domain in the α chain serves as the ligand-binding domain of several integrins. The domain can adopt two conformations, altering the affinity to the ligand. This domain is located at the distal part of the α chain, connecting to the β chain "propeller" region. In the high affinity state the metal ion dependent adhesion site (MIDAS), in the I-domain is occupied by a divalent metal ion (Harris et al., 2000). Only half of the α chains contain an I-domain and those lacking it form the ligand-binding site in association with the β chain. β Chains express an I-like domain that can serve as the main ligand-binding site (Qu and Leahy, 1995). The β I-like domain has three metal ion binding sites and in the low affinity state, all three sites are occupied by an ion.

Three main conformations of the hallmark leukocyte function-associated antigen (LFA)-1 (αL/β2) have been characterized. In the inactive state the ectodomains are closely associated with each other and the ligand-binding site is bent downwards facing the membrane. At this state, the intracellular domains are associated with filamin, connecting the integrin to the actin cytoskeleton. In the intermediate state, the head-domain extends, exposing the ligand-binding site. However, at this point the α and the β chains are still closely associated. When the integrin gets further activated, the binding of filamin is outcompeted by talin or the linker-protein 14-3-3. Phosphorylation of the intracellular threonine 758 is functionally important for the competition of talin and 14-3-3 binding. In the highly activated state the integrin is extended and the α and the β chain are separated. The phosphorylation of serine 1140 in αL intracellular tail is necessary for integrin activation and conformational changes (Gahmberg et al., 2009; Luo and Springer, 2006; Shimaoka et al., 2002). The phosphorylation status of the integrin can further mediate a crosstalk between different integrins where the activity of one integrin type regulates the activity of another (Uotila et al., 2014).

As a conclusion, there are several linking proteins that connect the integrins to the cytoskeleton. The anchoring to the actin filaments enables the trafficking and clustering of integrins. Clustering increases the avidity of the integrin. Clustering is meditated by a

change in the phosphorylation status in the cytoplasmic domain of the β chain, altering the interaction with cytoskeletal linker proteins (Boettiger, 2012).

Subunit combination		Gene	Name	Ligands
α1	β1	CD49a/CD29	VLA1	La, Co
α2	β1	CD49b/CD29	VLA2	La, Co, Th, Fn
α3	β1	CD49c/CD29	VLA3	La, Th, Fn
α4	β1	CD49d/CD29	VLA4	Th, VCAM-1, Os, Fn, MAdCAM-1
α5	β1	CD49e/CD29	VLA5	ICAM-5, Fn, Os, Fg
α6	β1	CD49f/CD29	VLA6	La
α7	β1	ITAG7/CD29		La
α8	β1	ITAG8/CD29		Fn, Os, Vi
α9	β1	ITAG9/CD29		VCAM-1, Os
α10	β1	ITAG10/CD29		La, Co
α11	β1	ITAG11/CD29		Co
αV	β1	CD51/CD29		Fn, Os, Vi
αL	β2	CD11a/CD18	LFA-1	ICAMs 1-5
αM	β2	CD11b/CD18	Mac1, CR3	ICAM-1, -2, Fg, iC3b, Factor X
αX	β2	CD11c/CD18	CR4	ICAM-1, -4, Co, iC3b, Fg
αD	β2	CD11d/CD18		ICAM-1, VCAM-1
αV	β3	CD51/CD61		Fn, Os, Fg, Vi, La, MMP-2
α11b	β3	CD41/CD61		Fn, Fg, Vi, Co
α6	β4	CD49f/CD104		La
αV	β5	CD51/ITGB5		Os, Vi, Fn
αV	β6	CD51/ITGB6		Fn, Os
α4	β7	CD49d/ITGB7		La, Fn, Os, VCAM-1, MAdCAM-1
αE	β7	CD103/ITGB7		E-Cadherin
αV	β8	CD51/ITGB8		Fn

Table 3. Integrins are heterodimers, consisting of one α chain and one β chain. The dimer forms the functional receptor that can bind ligands, listed in this Table. They include laminin (La), collagen

(Co), thrombospondin (Th), fibronectin (Fn), fibrinogen (Fg), osteopontin (Os), vitronectin (Vi) and mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1). Gene names are refered to as cluster of differentiation (CD). Receptor names include very late antigen (VLA), complement receptor (CR) and leukocyte function-associated antigen (LFA).

The main extracellular ligands to the integrins are listed in Table 3, modified from (Plow et al., 2000) and (Humphries et al., 2006). General ligands of the ECM and soluble proteins include laminins, collagens, fibronectin, fibrinogen, thrombospondin and vitronectin. Of these, all except laminin are bound through the RGD recognition sequence (Plow et al., 2000). Application of an RGD sequence containing peptide caused a decay in hippocampal LTP, indicating that integrin signalling is involved in early stage LTP (Xiao et al., 1991). Indeed, the integrins containing the α3 chain has been implicated in the consolidation of LTP (Kramár et al., 2002).

Microglia express a wide range of integrins, and their expression pattern is dependent on the state of activity (Milner and Campbell, 2003). Proinflammatory cytokines and ECM molecules associated with blood-brain-barrier breakdown generally upregulate integrin expression. In the brain, β1 integrins are the most abundant and mice devoid of it die shortly after birth with severe brain malformations (Graus-Porta et al., 2001). Microglia depend on β1 integrins in the phagocytosis of amyloid, implicated in Alzheimer's disease (Koenigsknecht, 2004). Proper integrin signalling is also required for a normal microglial response (process elongation and phagocytosis) to brain injury (Meller et al., 2017).

In addition to ECM proteins, one important group of ligands for the integrins are the intercellular adhesion molecules, ICAMs. The model integrin LFA-1 binds all the ICAM family proteins.

5. Intercellular adhesion molecule-5, redundant or essential?

The ICAMs belong to the immunoglobulin super family (IgSF) and five members of the family are known, ICAM-1 to ICAM-5. Common to the ICAMs are the extracellular Ig domains and they show similarities in ligand binding. The ICAMs mainly bind to leukocyte integrins and some ECM components, but may also interact with other CAMs. Most ICAM-family proteins are coded from genes on chromosome 19 and they are type 1 integral proteins, spanning the membrane once (Gahmberg, 1997; Kilgannon et al., 1998). ICAM-1 (CD54) has the widest expression pattern, ranging from leukocytes to fibroblasts. ICAM-1 has five Ig domains and it can bind LFA-1, Mac-1, CD43 and fibrinogen. ICAM-2 (CD102) is expressed on leukocytes, platelets and endothelial cells and it contains two extracellular Ig domains. Its function is mainly in the immune system, in which it interacts with LFA-1 and Mac-1. ICAM-3 (CD50) is apparently a leukocyte specific molecule, involved in the initiation of the immune response, whereas ICAM-4 (CD242) is confined to erythrocytes and was first discovered as the LW blood group antigen (Hubbard and Rothlein, 2000). ICAM-5 is the largest of all ICAMs, expressed in neurons in the mammalian CNS. Since ICAM-5 was first discovered in the telencephalon it was initially named telencephalin (TLCN)(Mori et al.,

1987). Further investigations based on sequence homology and integrin binding led it to be included in the ICAM family (Gahmberg, 1997). ICAM-5 is well conserved with over 80% of the amino acid sequence being identical in the mouse, in the rabbit and in humans. ICAM-5 shares 50% of the sequence with ICAM-1 (Yang, 2012). ICAM-1 is expressed as at least 6 different isoforms due to alternative splicing (Ramos et al., 2014). ICAM-5 might also exist in different isoforms, although less is known about it.

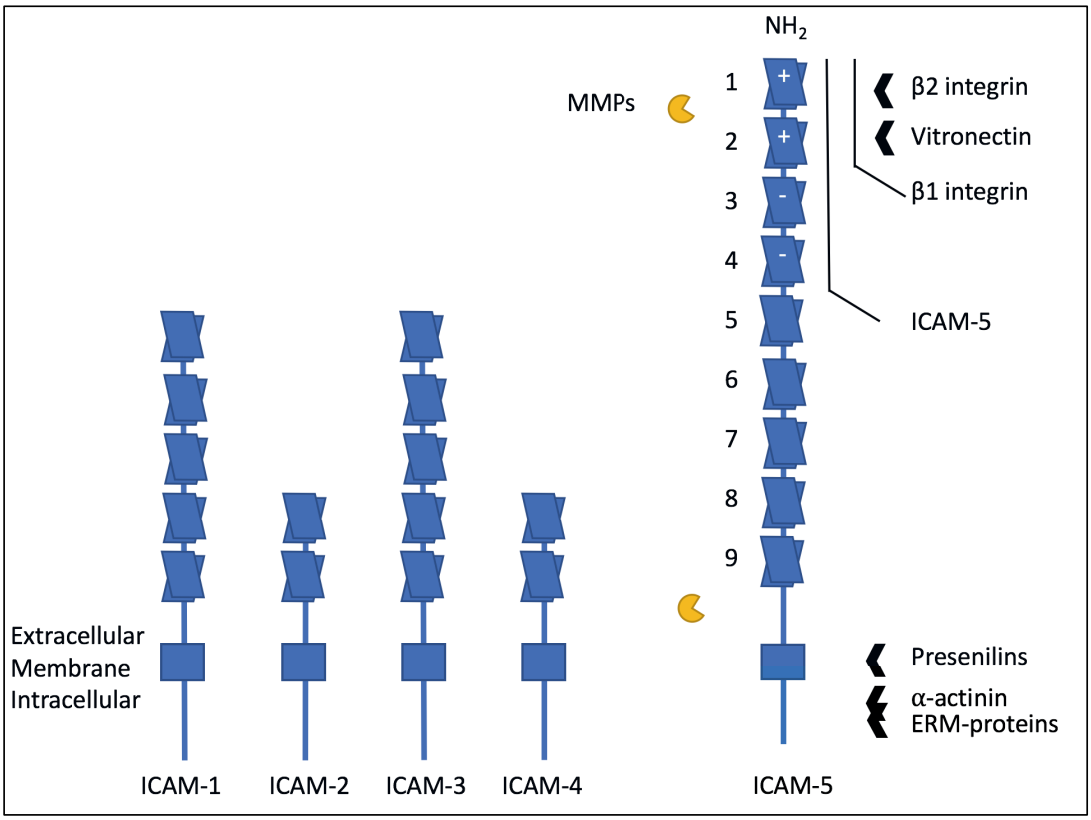

Figure 8. The intercellular adhesion molecule family. ICAM-5 is the most complex member in the family, with 9 extracellular Ig domains, D1-9. The two most membrane distal domains are basic (white plus), while the two following domains are acidic (white minus). The 4 NH_2-terminal domains mediate the homophilic adhesion pattern of ICAM-5. Domains 1-2 are also associated with integrin β1 and β2 binding, as well as with the ECM protein vitronectin. Extracellular ICAM-5 can be cleaved by MMPs (yellow figures), generating two soluble fragments. The cytoplasmic tail of ICAM-5 is bound by α-actinin and ezrin/radixin/moesin (ERM) proteins. The membrane-spanning region interacts with presenilins.

5.1. Neurons express the versatile adhesion molecule ICAM-5

ICAM-5 is a 130 kilo Dalton (kDa) type I integral glycoprotein with nine C2-type Ig domains. ICAM-5 Ig domains are formed by two anti-parallel β-sheets of 80-100 amino acids, associated by one or two disulphide bonds (Tian et al., 2000a; Yoshihara and Mori, 1994). The tandem Ig domains are named D1 to D9 in ascending order from the extracellular NH_2-terminal. D1-D5 are similar to the Ig domains of the other ICAMs, while D8-D9 are functional for ICAM-5 (Mizuno et al., 1997; Yang, 2012). The ectodomain contains 15 well preserved glycosylation sites, where a carbohydrate is linked to an asparagine. Asparagine-54 in D1 was shown to be functionally critical for ICAM-5, as the substitution of this amino acid resulted in failed filopodia formation and retarded cell growth in a cell culture system (Ohgomori et al., 2012). The 3D structure of Ig domains 1-4 has been determined (Recacha et al., 2014; Zhang et al., 2008a). The crystal structure revealed that the basic D1-D2 domains (arginine, lysine and histidine) bind the acidic residues (aspartic acid and glutamic acid) in domains 3-5 in a direct or possibly in a zipper like fashion (Recacha et al., 2014). This homophilic adhesion of ICAM-5 is unique within the ICAM family and functionally important in neurite sprouting and dendritic arborisation (Tian et al., 2000b).

The expression of ICAM-5 emerges soon in postnatal mice and prenatally at gestational week 29 in humans. The expression increases rapidly and is sustained throughout adulthood. It is expressed by spiny glutamatergic neurons, especially in the hippocampus and in the cortical molecular layer V, and it is absent from GABAergic neurons (Arii et al., 1999; Yang, 2012; Yoshihara and Mori, 1994). The confinement to the somatodendritic region is directed by the cytoplasmic domain and one amino acid, phenylalanine-905, is of particular importance. Deletion of this amino acid results in a diffuse distribution of the protein (Mitsui et al., 2005). The expression is particularly prominent in filopodia.

ICAM-5 knock out (KO) mice live and reproduce normally. Brain wiring and the general synapse anatomy is normal, although LTP and reference memory are improved (Nakamura et al., 2001). At the cellular level, filopodia appear less dense compared to wild type and maturation into mushroom-shaped spines is accelerated, observed as an enlargement of the spine heads (Matsuno et al., 2006). On the other hand, ICAM-5 overexpression causes a dramatic decline in the ratio of mature spines to filopodia, due to an increase in filopodia density. The same pattern is seen when ezrin is overexpressed (Matsuno 2006, Furutani 2007). Hyperactivation or overexpression of the Ras-Akt signalling pathway or Polo-like kinase 2, respectively, has similar effects as ICAM-5 on accelerated filopodia formation and decreased spine maturation (Yoshihara et al., 2009).

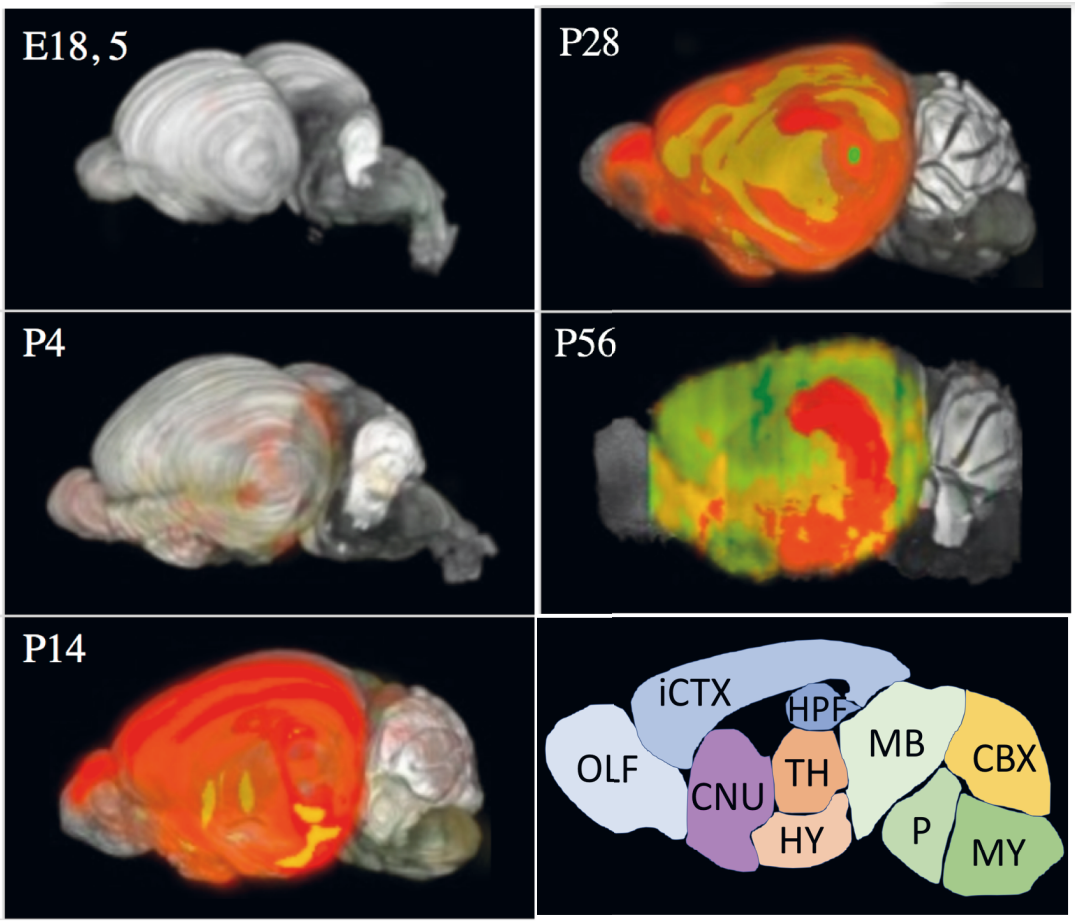

Figure 9. In rodents, the expression of ICAM-5 appears around birth, and it is strongly upregulated during early postnatal life. The peak of ICAM-5 expression coincides with the most active time of synaptogenesis at around P14. In adults, ICAM-5 remains highly expressed in the hippocampus (P56, intensely red area). Mouse brain, grey: no expression, green, low expression, red: high expression. E: embryonic day, P: postnatal day. Image credits: Allan institute. The lower right corner represents a schematic overview of the main brain areas of the a P56 mouse brain in a sagittal section. OLF: olfactory areas, iCTX: isocortex, HPF: hippocampal formation, CNU: central nuclei, HY: hypothalamus, TH: thalamus, MB: midbrain, P: Pons, MY: medulla oblongata, CBX: cerebellum. The areas in shades of blue make up the cortex, blue and purple is the cerebrum, brown areas is the interbrain, brown and green areas make up the brain stem.

5.3. The ambidextrous ICAM-5 regulates both neuronal and leukocyte functions

ICAM-5 is a multifunctional molecule with its heritage in the immune system and its residence in the nervous system. It regulates the T cell response and it is thought to be important through the lifespan of a glutamatergic neuron, from neurite sprouting to synapse formation and transmission.

ICAM-5 interacts with intracellular proteins that are associated with the localization and function of ICAM-5. It is linked to the actin cytoskeleton through α-actinin. This binding has been mapped to the residues 857-861 in ICAM-5, an area which talin and filamin are unable to bind to. The binding site of ERM-proteins partially overlap that of α-actinin in the ICAM-5 cytoplasmic tail. The association to the cytoskeleton was proven to be functionally important for neurite outgrowth (Nyman-Huttunen et al., 2006).

During the filopodia-to-spine transition, there is a large turnover and reorganisation of membrane associated proteins. ICAM-5 is one of the proteins that undergo locational change in the spine during the maturation process. It is expressed abundantly in the filopodia but is almost absent from mature spines. This protein is considered a negative regulator of spine maturation since the protein is concentrated in the early filopodia structures, serving as an important factor for filopodia formation and maintenance (Matsuno et al., 2006; Tian et al., 2007).

When spines mature, ICAM-5 is removed, either by shedding, internalization or trafficking (Raemaekers et al., 2012; Tian et al., 2007; Yoshihara et al., 2009). The guanine exchange factor 6A, EFA6A, serves as an activator for the small GTPase ADP-ribosylation factor 6, ARF6, and is required for the internalization of ICAM-5. When ICAM-5 and EFA6A were co-expressed *in vitro*, ICAM-5 lost the enrichment in filopodia and became accumulated in intracellular vesicles. ICAM-5 must dissociate from the actin cytoskeleton after which endocytosis mediated by ARF6 becomes possible. The dissociation is mediated by Rac1, which releases ICAM-5 from the ERM proteins. Additionally, ICAM-5 has been shown to co-localize with lipid-raft micro-domains (Raemaekers et al., 2012).

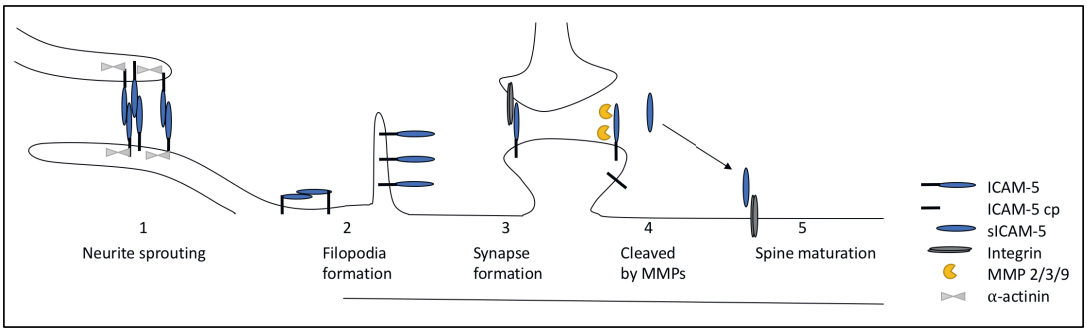

Figure 10. ICAM-5 is involved in neuronal maturation, from neurite sprouting to spine maturation. 1) Homophilic binding and cytoskeletal anchorage through α-actinin is important in neurite sprouting. 2.) ICAM-5 induces filopodia formation. 3) ICAM-5 stabilizes synaptic contacts by binding to α5/β1 integrins on the axonal terminal. 4) Glutamatergic transmission induces an MMP mediated shedding of ICAM-5 that generates soluble fragments of the extracellular domain, as well as the short intracellular tail. 5) The soluble fragment of ICAM-5 is bound by nascent β1 integrins, inducing spine maturation.

ICAM-5 is cleaved by MMP -2, -3 and -9. The MMPs are zinc-dependent endoproteases that cleave several targets, among them ECM components and adhesion molecules. These proteases can be secreted by microglia, T cells or neurons and their function vary from neuronal development to injury (Milward et al., 2007). MMP-9 can be translated in the spine and activated in the synaptic cleft. Studies have shown that MMP-2 and -9 can be stored in vesicles in synaptic structures and secreted upon calcium-dependent stimulation (Kean et al., 2009). MMP-9 is known to be involved in learning and memory formation, and the activation of MMP-9 is required for long-term memory (Nagy et al., 2007). Activation by NMDA induces MMP secretion that leads to the cleavage of the ICAM-5 ectodomain at the first Ig domain and close to the membrane in maturing spines (Tian et al., 2007). ICAM-5 is solubilized in LTP and the soluble fragment enhances excitability in neuronal networks (Conant et al., 2010; Lonskaya et al., 2013; Niedringhaus et al., 2012; Ning et al., 2013). The cleavage products may influence spine morphology through homophilic binding to intact ICAM-5 molecules (Tian et al., 2007) or heterophilic binding to β1 integrins and their function in cytoskeleton modification (Conant et al., 2010). ICAM-5-β1 integrin signalling induces phosphorylation of cofilin, an actin severing and depolymerizing protein, thus inducing cofilin inactivation. ICAM-5-β1 integrin signalling induced cofilin inactivation promotes dendritic spine maturation (Conant et al., 2011).

At synaptogenesis, ICAM-5 interacts with presynaptic β1 integrins, mainly α5β1. The binding forms at early contacts and prevents spine maturation. The binding of ICAM-5 to β1 integrins reduces the shedding of ICAM-5 and therefore slows spine morphogenesis (Ning et al., 2013). In this study, it was found that neurons devoid of ICAM-5 have an abnormally high number of functional synapses and an elevated rate or glutamatergic release probability, suggesting a regulatory role for ICAM-5 on presynaptic function as well.

The ability of ICAM-5 to induce actin reorganization was also characterized in the binding to vitronectin. Vitronectin is a component of the ECM and when it binds to ICAM-5, intracellular ERM proteins are phosphorylated and PIP_2 accumulates. The subsequent recruitment of filamentous actin results in spine morphogenesis and the formation of phagocytic cup-like structures. The exact function of these structures remains elusive. The deletion of D2 in ICAM-5 completely abolished the binding (Furutani et al., 2012).

T cells patrol the brain parenchyma and can bind to hippocampal neurons through LFA-1 (Tian et al., 1997, 2000a). The glutamate-37 in ICAM-5 D1 is important for binding to the I domain of LFA-1. This interaction is interrupted by function blocking antibodies or by the deletion of these domains. The binding is promoted through an allosteric regulation of an α helix in the LFA-1 I-domain (Zhang et al., 2008a). Mainly through this interaction, ICAM-5 can reduce the T cell receptor mediated activation, seen as a reduction in activation markers in naïve T cells. Soluble ICAM-5 was further shown to induce the expression of TGF-β and IFN-gamma. Whereas ICAM-1 is upregulated when cells were stimulated with inflammatory cytokines, ICAM-5 is not. While soluble ICAM-1 is known to be pro-inflammatory, ICAM-5 was found to have the opposite effect (Tian et al., 2008). In addition, a study showed that also microglia react to ICAM-5 by forming extensions called lamellopodia and clustering of β2 integrins *in vitro* (Mizuno et al., 1999).

ICAM-5 is believed to be involved in diseases such as encephalitis, MS and epilepsy, due to the fact that the level of soluble ICAM-5 was abnormal (Borusiak et al., 2005). Activated T cells induce MMP activation and subsequently increase the ICAM-5 cleavage (Tian et al., 2008). Alzheimer's disease (AD) is characterized by the accumulation of amyloid-β debris, forming plaques in the neuronal parenchyma. Even though the overall expression of ICAM-5 in the neocortex of AD patients is reduced, the ICAM-5 expression in the neurites of surviving neurons and in the vicinity of amyloid-β plaques is up-regulated (Hino et al., 1997). The membrane-spanning region of ICAM-5 associates with presenilins, which are part of the gamma-secretase complex. This complex is responsible for the formation of amyloid β from APP, implicated in AD (Annaert et al., 2001). Precenilin-1 is not involved in the proteolysis of ICAM-5, but it plays a role in the autophagic turnover of ICAM-5 (Esselens et al., 2004). Studies moreover suggest that ICAM-5 might be involved in perineural tumour formation and metastatic invasion (Maruya et al., 2005), as well as in the virulence of human immunodeficiency virus, enterovirus D68 and herpes simplex virus type-1 (Tse et al., 2009; Wei et al., 2016; Yuan et al., 2017).

In conclusion, ICAM-5 plays important roles throughout the lifespan in various ways. During early neuronal development, it induces neuronal sprouting. Then, ICAM-5 drives the formation of filopodia and regulates synaptic maturation. During immunological challenges and in disease, ICAM-5 again has a role to play.

HYPOTHESES AND AIMS OF THE STUDY

Hypotheses

ICAM-5 regulates synaptic maturation through an α-actinin dependent mechanism. ICAM 5 is bound to microglia by β1 and/or β2 type integrins and it is involved in the regulation of microglia functions in synaptic pruning and immunity.

Aims

1. To characterize the molecular mechanism of how ICAM-5 regulates synaptic maturation
2. To study if ICAM-5 interacts with microglia
3. To assess whether the ICAM-5-microglia interaction is conducted through β1 or β2 type integrins
4. To elucidate the role of ICAM-5 as a I) regulator of microglia activation and II) regulator of synaptic pruning by microglia
5. To identify novel binding partners for ICAM-5

MATERIALS AND METHODS

The material and methods used in this study are described in detail in the original publications, and hence provided here in the form of a list with references to the original publications and a brief description below. Methods with unpublished data are described in more detail.

	Method	Original publication
Molecular		
	Protein expression and purification, *E. coli*	III, IV
	Protein expression and purification, COS cells	IV
	Immunofluorescence staining	I, II, IV
	Western blot	III, IV
	SDS-PAGE	III, IV
Cell culture		
	Mammalian cell lines	III, IV
	Mouse primary neurons	II - IV
	Mouse primary microglia	IV
Cell assays		
	Cell stimulation / protein binding	I, III, IV
	Cell adhesion assays	IV
	Bead-recruitment assay	IV
	Phagocytosis assays	IV
	RTCA iCELLigence	IV
Protein assays		
	Protein competition assay	III
	Co-immunoprecipitation	III, IV
	Pull-down assays	IV
	Mouse brain homogenisation	IV
Imaging		
	Confocal microscopy	I, IV
	Epi-fluorescence microscopy	I, IV
	Transmitted light microscopy	IV

Fluorescence activated cell sorting, FACS

The phagocytosis experiment was verified by FACS. Microglia were isolated from the glia mix and divided to at least 300 000 cells in 500 ul culture medium per sample. Recombinant proteins were added to a final concentration of 100nM and 10ul phagocytosis beads that had been diluted 1:10 from the original vial was added. The cells were incubated for 1 h at 30°C with end-over-end rotation and then analysed by FACS. Untreated cells and beads only were analysed as controls. The bead content in the cells was analysed by BD LSR 2 or BD Fortessa Flow Cytometer and the software Facs Diva 8.0 (all by BD Biosciences). The live cell population was first gated and under this gate, the fluorescence corresponding to endocytosed beads, was measured. No free beads were found in this gate due to size exclusion.

Proteome profiler, mouse XL-Cytokine array

The regulatory role of ICAM-5 on the microglia inflammatory profile was analysed by ELISA (publication VI) and by the Proteome profiler, mouse XL-cytokine array (Biotechne). For the ELISA assay, purified primary microglia cultures were treated with 10 nM LPS and soluble, recombinant proteins and the culture media were collected and analysed. The regulatory role of ICAM-5 was also analysed by the cytokine array. For this purpose, mixed glia cultures were prepared from P6 C57bl6 cortices by enzymatic digestion and plating on poly-L-lysine coated 48-well tissue culture plates. At 10 days *in vitro* (DIV), the mixed glia cultures were treated for 24 h with wild type (WT) or ICAM-5 KO cortical neuron-conditioned medium (HBSS supplemented with 20 μM NMDA and 1,8 mM $CaCl_2$ for 6 h in the cell culture incubator) that had been supplemented with 10 nM LPS. The medium from two parallel wells was collected and centrifuged to remove cellular debris and then analysed by the XL-cytokine array kit, according to the manufacturer's instructions.

BDNF protein expression analysis

The prefrontal cortices of P6 WT or ICAM-5 KO mice (N=10 from two litters, 4+6) were collected and homogenized in 2% RIPA (150 mM NaCl, 50 mM Tris, 10 mM $MgCl_2$, 5 mM EDTA, 1% Triton-X 100, 1% NP-40, pH 7.8) using a piston. The homogenates were cleared by centrifugation. The total protein concentration was measured by the Pierce BCA protein assay kit (ThermoFisher scientific) and 40 μg was analysed by Western blot.

Pull down assay and mass spectrometry

In order to find novel binding partners for ICAM-5 in microglia, ICAM-5 D1-2-Fc was coupled to protein G sepharose. Human IgG was used as control. One μg of protein was coupled to 10 μl of sepharose slurry for 2 h at +4°C. The coupled sepharoses were then washed twice in phosphate buffered saline (PBS). Cells of the murine microglia cell line BV-2 were cultured until 80% confluent in 10-cm tissue culture plates and then washed 3 times in cold PBS and lysed. Lysates were prepared by incubating the cells in 1 ml per plate, 2% RIPA buffer for 20 min on ice. Lysates were cleared by centrifugation and the corresponding lysate of one plate per sample was pooled and pre-cleared by incubating at least 2 h at +4°C with 20 μl protein G slurry per ml lysate. The pre-cleared lysate was divided over the ICAM-5 D1-2-Fc or IgG coupled sepharose and incubated at +4°C over night. The unbound lysate

was removed and the sepharose samples were washed twice in 2% RIPA buffer and twice in PBS. Then the protein complexes were eluted from the sepharose with 0.2 M glycine, pH 2.8. 10% of the sample was analysed by SDS-PAGE and silver stain, and the remaining sample of the ICAM-5 pull down was analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the proteomics unit, University of Helsinki.

Phage display and next generation sequencing

The two pools of eluted phages were combined and used as templates to run 10 cycles of PCR. The primers were designed to amplify the region with the random amino acids and to incorporate the indexes and adapters needed for next generation sequencing with Illumina MiSeq (Forward primer: 5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACAC GACGCTCTTCCGATCTGCAAGCTGATAAACCGATACAAT 3' and reverse primer: 5' CAAGCAGAAGACGGCATAC GAGATTAGCTGGGGTGACTGGAGTTCAGACGTGTGCTCTT CCGATCTCCCTCATAGTTAGCGTAACGATCT 3', from Oligomer Oy) The PCR products were handled and analyzed at the sequencing and genomics lab at the University of Helsinki. The identified sequences were ranked by the number of reads and sequences with less than 200 reads were discarded. The number of reads corresponds to the abundance of the amplified phage. The sequences were analyzed using the 4 GAP software (Bonfield et al., 1995) were the region with the random amino acids were extracted as a protein sequence. This sequence was then analyzed by BLAST to find matching proteins. The most relevant hits are listed in Table 4.

RESULTS

1. ICAM-5 regulates spine maturation through α-actinin

In developing spines, ICAM-5 inhibits the maturation process. We found that the competition between ICAM-5 and GluN1 in binding to α-actinin might explain this effect (II). The binding region of ICAM-5 partially overlapped with that of GluN1 to α-actinin rod domain 2. In a competition assay, ICAM-5 showed a higher affinity for α-actinin, as compared to GluN1, indicating an advantage for ICAM-5. Accordingly, ICAM-5 outcompetes GluN1 in the binding to α-actinin *in vivo* in P14 mice. At this developmental stage, there is robust synaptogenesis in mice and ICAM-5 is found in dendritic protrusions (Kelly et al., 2013).

In filopodia, α-actinin is dynamic and localizes mainly to the neck, where ICAM-5 is abundant. As NMDA receptor activation causes ICAM-5 to be degraded, the intracellular tail dissociates from α-actinin, allowing re-localization and clustering of α-actinin to the spine head and root and an increased association to GluN1. ICAM-5 regulates α-actinin through the cytoplasmic tail and the intact molecule is needed. The general MMP inhibitor, GM6001, completely abolished the MMP and NMDA mediated shedding of ICAM-5, which also prevented ICAM-5 from dissociating from α-actinin. Similarly, neurons lacking ICAM-5 or cultured neuronal cell lines that express a truncated form of ICAM-5, lacking the cytoplasmic tail, showed increased association between α-actinin and GluN1. The clustering of α-actinin is associated with f-actin, indicating that ICAM-5 regulates actin dynamics through α-actinin.

2. ICAM-5 is bound by microglia and downregulates phagocytosis and adhesion

We identified a function for the cytoplasmic tail of the truncated ICAM-5 in synaptic maturation, and we were further curious to see if the corresponding extracellular fragment of ICAM-5 would have a biological effect as well. Soluble ICAM-5 is bound by microglia and integrins are partially responsible for this interaction. Glutamate-37 in ICAM-5 is important for the binding of ICAM-5 to β2 integrins, less so to β1 integrins (IV). Glutamate-37 was predicted to be critical for LFA-1 binding (Zhang et al., 2008a), which we could confirm.

We identified an inhibition of phagocytosis by microglia as a result of ICAM-5 stimulation. Primary microglia were allowed to internalize small latex beads in a medium supplemented with soluble, purified proteins in a phagocytosis assay. The number of beads that had been phagocytosed in 1 h was analysed manually in micrographs, or by fluorescently activated cell sorting (FACS, Figure 11). Both approaches produced similar result; soluble ICAM-5 reduces the number of beads that microglia internalized. The effect was more dramatic when cells were kept in culture, as compared to cells that had been in solution, due to rotation during

the experiment for the FACS analysis. Additionally, soluble ICAM-5 reduced the static adhesion of microglia to purified iC3b, a component of the complement system (IV).

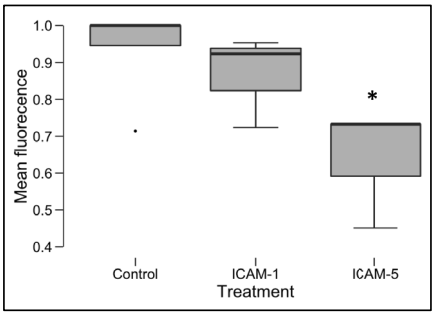

Figure 11. Soluble ICAM-5 significantly (*P = 0,042) reduced the phagocytosis of latex beads by microglia, measured by FACS. Samples were normalized within experiments and the control without additional protein was set to 1. Mean fluorescence reflects the number of bead per cell as the cell population was gated and beads are highly fluorescent. No free beads were found in this gate, due to size exclusion.

3. ICAM-5 promotes an anti-inflammatory response in LPS stimulated microglia

Since ICAM-5 has been shown to regulate the inflammatory state of peripheral T cells, we investigated the regulatory role of ICAM-5 on LPS-stimulated microglia. Isolated primary microglia treated with LPS and one of the following purified recombinant protein ICAM-1, ICAM-5 D1-9 or human IgG or vehicle all upregulated the expression of inflammatory cytokines TNF-α and TGF-β, except for cell treated with soluble ICAM-5 D1-9. Compared to the other samples, ICAM-5 stimulated the cells to upregulate the anti-inflammatory IL-10, while downregulating TNF-α and TGF-β. CCL-5 was also analysed, but the differences were less obvious even though ICAM-5 seems to slightly downregulate this proinflammatory cytokine (IV).

We further screened for the effect of ICAM-5 on the cytokine/chemokine profile in a more general setting. Primary cultures of WT or ICAM-5 KO neurons were treated with NMDA in a paradigm to create a conditioned medium rich in soluble ICAM-5 to be supplemented with LPS and used to treat mixed glia/neuronal cultures. This screen suggests that several other cytokines are associated with the anti-inflammatory phenotype of microglia. Strikingly, ICAM-5 induced the secretion of IL-1ra, while suppressing MMP-3, MMP-9 and fractalkine. Most strikingly, ICAM-5 induced the secretion of angipoietin-2, a mostly pro-angiogetic, but also considered a proinflammatory factor (Scholz et al., 2015).

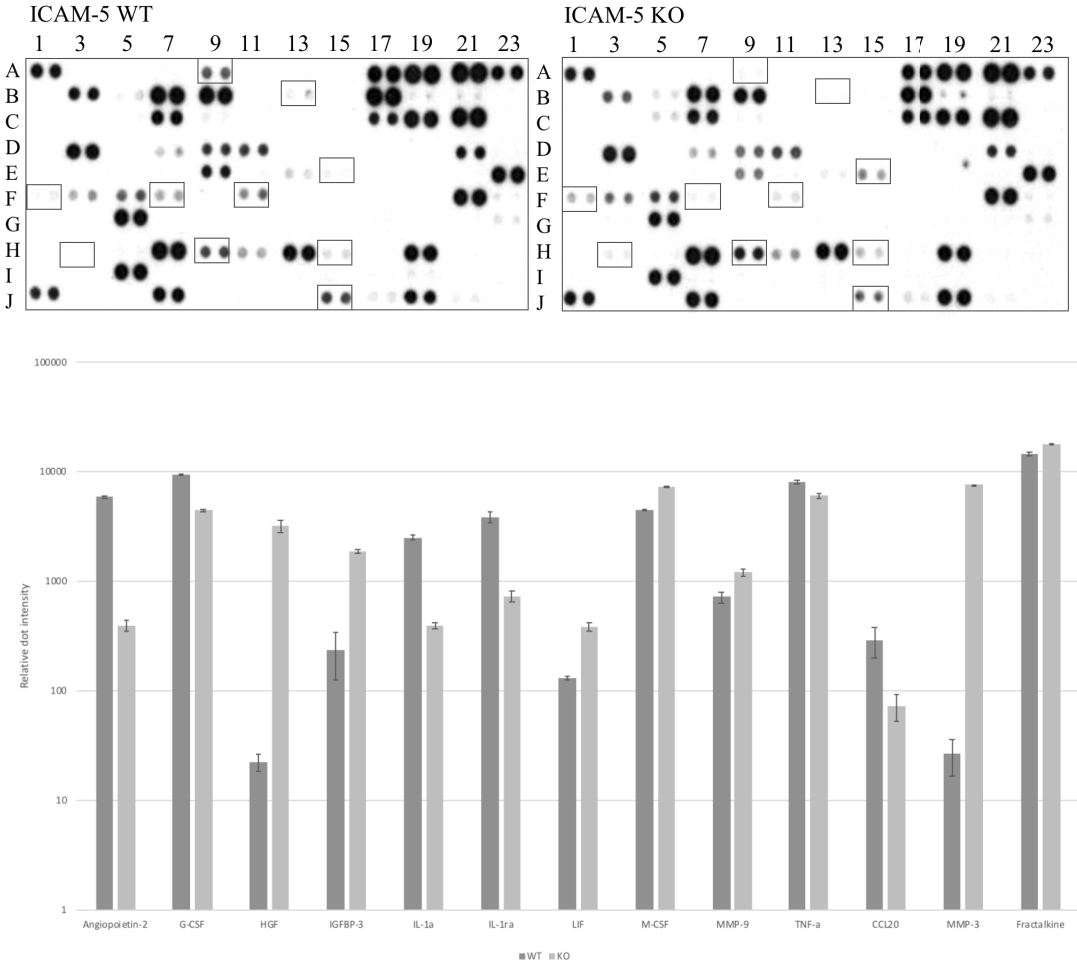

Figure 12. Endogenous soluble ICAM-5 from neuronal cultures generates a different cytokine profile, as compared to conditioned culture medium from ICAM-5 KO neurons in a cytokine array of mixed glia cultures treated with neuron conditioned culture medium supplemented with LPS. Error bars are +/- SD of the mean relative dot intensity calculated from the western blots above. A9: Angiopoietin-2, C17: Fractalkine, E15: HGF, F1: IGFBP-3, F7: IL-1a, F11: IL-1ra, H9: M-CSF, J15: TNF-α.

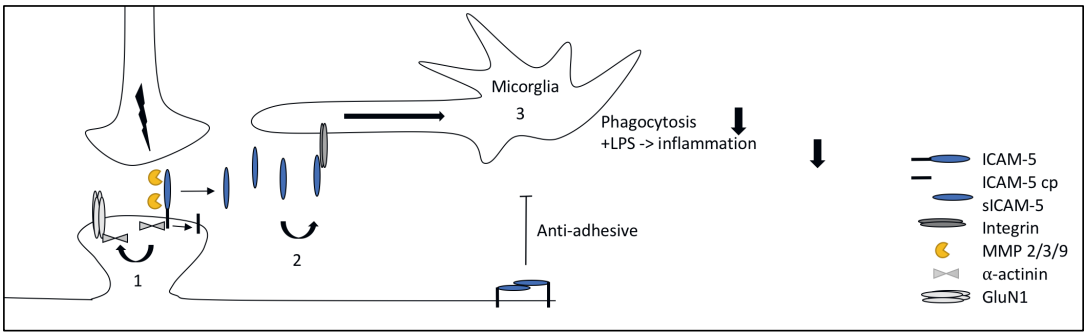

Figure 13. Overview of the main findings. 1. NMDA treatment of neurons induces cleavage of ICAM-5, and as a consequence, the intracellular tail of ICAM-5 dissociates from α-actinin. This enables GluN1 receptors to be recruited. 2. The soluble fragment of ICAM-5 is bound, at least partly, by integrins on microglia, resulting in reduced phagocytosis. Microglia efficiently adhere on ICAM-5 D1-2, while D1-9 is anti-adhesive. LPS-stimulated microglia are tilted towards a less proinflammatory phenotype if they are co-stimulated by soluble ICAM-5.

4. ICAM-5 regulates BDNF in the prefrontal cortex of P6 mice

To explain the observed effect of ICAM-5 on spine maturation, where ICAM-5 acts as a negative regulator of the maturation process, we studied the BDNF protein content by western blot analysis. In the prefrontal cortices of 6-day-old mice, BDNF is up-regulated in ICAM-5 KO. BDNF is expressed as a mature form at around 15 kDa, a pro- and pre-pro form at approximately 25-32 kDa and a dimer of the pro-form at around 64 kDa. In figure 14, all bands visible by blotting for BDNF are shown and samples from three animals from each genotype is blotted, along with the corresponding β-actin band as a loading control. All of these were more strongly expressed in the knock out animals ($P<0,01$). There did not seem to be any difference between sexes (data not shown).

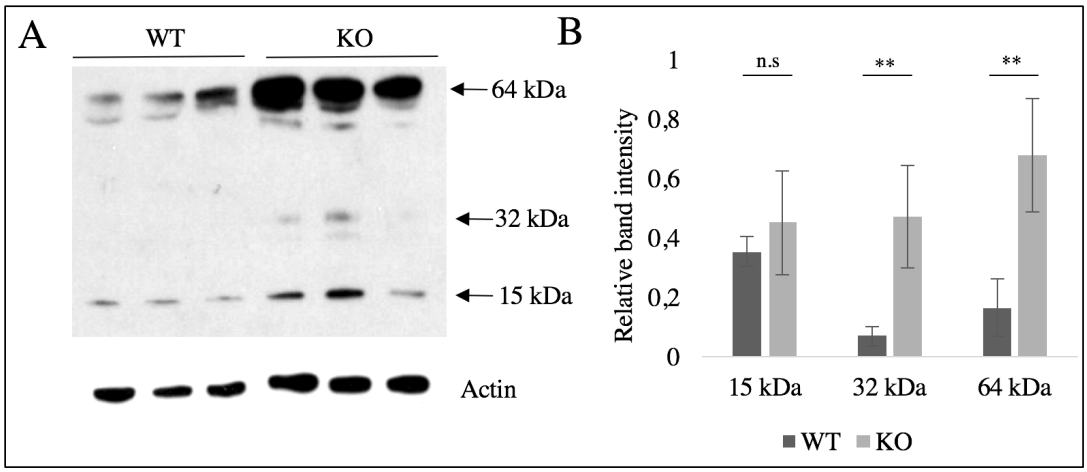

Figure 14. ICAM-5 knock out mice have more immature BDNF in their prefrontal cortices at P6, as compared to wild type mice. A. A BDNF western blot of brain homogenates, three WT animals and three ICAM-5 KO animals. BDNF is produced as a pro-form of 32 kDa that is cleaved to a 15 kDa mature form. Probably, the 64 kDa band is a dimer of the pro-form. The relative band intensities were normalized to the corresponding actin band and quantified in B. ** $P < 0,01$, not significant (n.s)

5. ICAM-5 might regulate neuronal- and microglia functions through novel ligands

Since the co-immunoprecipitations of ICAM-5 and integrins led us to believe that additional receptors for ICAM-5 might exist, we performed a phage display in order to find ICAM-5 D1-2 binding peptides. The identified proteins that are relevant for ICAM-5 are listed in table 4. The most abundant peptide had a highly reliable match in APLP2, with eight identical amino acids. This corresponds to amino acids 271-279 in the acidic part of the APLP2 ectodomain. Importantly, integrin αL and αX polypeptides were also identified. Sialoadhesin is of interest since it is a macrophage specific adhesion molecule (O'Neill et al., 2013) and it was also identified by LC-MS/MS from the pull-down experiment. The silver stained SDS-PAGE gel of the pull-down analysis suggests that there might be several receptors for ICAM-5 on BV-2 cells, based on the number of bands, figure 15. Components of the complement system was picked up by both the phage display and the pull-down assay. Of these, C1q, with the amino acids sequence YYFTF is of high interest, since it is the initiating factor of the complement cascade.

Sequence	Nr. of reads	Potential ligands	Peptide match
R D Y Y Y D T F	24626	Amyloid precursor like protein-2 (APLP2)	RDYYYDTF
QTLAEGA	20898	Laminin	Q-LAEGA
V S F V T G A	19993	Type XV collagen	VSFVTG
C T V F T P D V	5381	ITGAX protein	VFTPD
I S L V S Y A	2985	Sialoadhesin	ISL-VSYA, SLVS
		Adhesion G-protein coupled receptor G1	SLVSY
I S F E A G A	2848	Glioma-expressed antigen 2	ISFE-GA
W A L A D E V	1973	Down syndrome cell adhesion molecule 1	WAL-DE
		Intersectin 1	WALAD
I R L S D F V	1211	Spastin	IRLSDF
A I F A S G	1124	Syntaxin	AIFASG
		ITGAL protein	IFAS
E I L S A G V	706	Complement component C5	EILSA
D S D H Y Y F T F	622	Complement C1q	YYFTF
		Sodium channel, voltage-gated	HYYFT
		Monocyte-macrophage NaV1.6	HYYFT
V M L A P D V	301	Collagen, type VII	MLAPD
V S F V P E V	247	Mucin 5B	VSFVPEV

Table 4. ICAM-5 D1-2 binding peptides and their corresponding proteins, identified by phage display. The sequence of the phage as well as the corresponding sequence in the identified protein is listed. Number of reads corresponds to the relative abundancy of the peptide in the panned pool of the phage library. Proteins of particular interest are indicated in red.

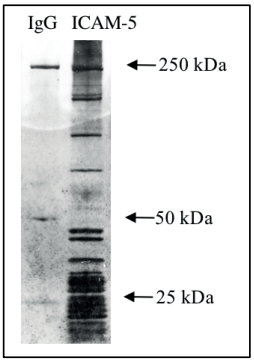

Figure 15. Silver stained SDS-PAGE of the pull-down assay where ICAM-5 D1-2 was used as bait reveals that several proteins from the BV-2 lysate were found in the complex. In a pilot experiment, the same sample was analysed by mass spectrometry, suggesting that sialoadhesin, complement C3 and galectin-1 and -3 could bind ICAM-5.

DISCUSSION

The robust shedding of ICAM-5 is a costly process and must hence be important. This work collectively shows that both the intracellular and the extracellular fragments of ICAM-5 have relevant and diverse functions. ICAM-5 has proven to be an ambidextrous molecule contributing to both synaptic plasticity and immunology. In the past decade, microglia research has been an accelerating train that has laid ground-breaking discoveries in its path. With this work, we contributed with some pieces in the puzzle in how microglia interact with neurons, how neurons regulate various aspects of microglia function, and how synapse formation is regulated.

α-Actinin is a dimer that can hinge actin filaments and anchor various other proteins simultaneously. It is a master hub in the postsynaptic terminal, docking adhesion molecules and receptors. ICAM-5 anchorage to the actin cytoskeleton is important and imposes a competition in the binding of other molecules. Competition like this is pivotal in developmental switches and processes where events must be carefully temporally regulated. We identified a competition between ICAM-5 and GluN1 in the binding to α-actinin in maturing spines. Furthermore, by inhibiting the clustering of α-actinin in spine heads and keeping it dynamic, we believe ICAM-5 inhibits spine maturation. When ICAM-5 is degraded, it dissociates from α-actinin, allowing it to relocate, possibly together with GluN1 receptors, to the spine head. Here, the anchorage of NMDA receptors to the actin cytoskeleton is functionally important (Wyszynski et al., 1997).

Shedding of the ectodomain of ICAM-5 releases the intracellular tail from α-actinin. This could be an interesting novel mechanism of how changes to the extracellular part of the molecule elicits intracellular signals. Since inhibiting the proteolytic function of MMPs abolished the effect of NMDA, it implies that it is probably not intracellular signalling from the NMDA receptor that releases ICAM-5 from α-actinin. Instead, ICAM-5 might be rapidly degraded after it has been cleaved, as has been shown previously (Raemaekers et al., 2012), or it might be due to the loss of trans-state polymerization due to the release of the ectodomain.

Calmodulin also competes for with GluN1 in the binding to α-actinin, reducing GluN1 efficacy (Merrill et al., 2007). However, this interaction is probably more important in mature synapses, since it has been suggested to function as a protective switch, reducing excitotoxicity. The novel interaction between ICAM-5, GluN1 and α-actinin might be important in slowing the maturation process in young spines, to prolong critical developmental windows.

Future efforts should be focused on deciphering the network of interactions where different isoforms of α-actinin and the various binding partners is studied simultaneously and at different developmental stages.

Integrins bind their ligands with relatively low affinity, which reflects their function. Hence, it proved very challenging to identify the integrin receptors for ICAM-5. The bead adhesion assay provided some clues that both β1 and β2 integrins are involved in the binding. Further, they seemed to be recruited at different times, β2 followed by β1, in microglia. The phage display mapped the ICAM-5 D1-2 binding sequence to the I-domain of LFA-1 α subunit. This is in line with previous findings, that the I-domain is the ligand binding domain. Further interesting candidates were found in this experiment, as seen in Table 4. These include the CR4 (integrin αX), the APLP2 and complement components. Moreover, we obtained indicative results by mass spectrometry that sialoadhesin, complement C3 and galectins might bind ICAM-5. However, this experiment needs to be repeated and future experiments could rather be conducted as a co-immunoprecipitation of ICAM-5 from mouse brain, instead of using BV-2 lysate and purified recombinant ICAM-5. APLP2 has been less studied than other members in the APP family, although it is known to function as an adhesion molecule in synapses. It remains to be seen, whether ICAM-5 and APLP2 collaborate in the formation and maturation of synapses. The identification of complement components is not only intriguing considered the recent excitement over their involvement in microglia pruning on a fundamental level, but also from a therapeutic perspective. Excessive pruning by microglia has been suggested to be involved in various pathological conditions including neurodegenerative disorders (Hong et al., 2016; Johnson and Stevens, 2018). A synthetic peptide that would interrupt this aberrant function could be a valuable tool. Phagocytosis is another aspect of the pruning process and we found that ICAM-5 reduces this endocytic function of microglia. It might not be related to pruning, but it is certainly appealing to speculate about it. Since ICAM-5 inhibits spine maturation, we believe that it could prolong critical windows in neuronal wiring. Maybe the reduced phagocytosis by microglia is one mode of how ICAM-5 functions as a don't-eat-me signal, protecting immature spines during early development.

Microglia were found to internalize ICAM-5 in all forms, the endogenous ICAM-5, shedded from neurons and recombinant D1-2-Fc or D1-9-Fc. To control for the Fc tag binding to Fc receptors on microglia, ICAM-5 D1-2 was subcloned into a His-V5 containing expression vector. This recombinant protein was efficiently internalized by BV-2 cells, as was the D1-4 construct from which the Fc tag had been cleaved (data not shown). The recombinant ICAM-5 proteins clearly implied a versatile effect of ICAM-5 in the adhesion assay. D1-9-Fc induces rapid and robust clustering of microglia and BV-2 cells, while D1-2 is very adhesive. This experiment demonstrates that ICAM-5, as a large molecule, can regulate its properties by conformational change or polymerization. When the integrin-binding D1-2 is exposed, binding to microglia is strong. However, the full molecule can polymerize and hence mask potential integrin binding sites or fold back on itself to exert the same effect.

It is possible that posttranslational modifications, such as polysialyation, to the more rostral Ig domains could cause the anti-adhesive effect. NCAM can be polysialylated at Ig domain 5, which renders it repulsive (Johnson et al., 2005). This effect seen by the full ectodomain of ICAM-5 could explain why microglia tend not to contact the dendritic trunk, where ICAM-

5 is abundantly expressed. Variation in post-translational modification of the purified proteins might explain the contradiction in our results, compared to others where they found that microglia spread on ICAM-5 (Mizuno et al., 1999).

Developmental endothelial locus (Del)-1 is a molecule that has been identified to modify adhesion in a manner that seems similar to our results for ICAM-5. Del-1 can bind integrins, but does not elicit the intracellular signalling cascade, characteristic for outside-in signalling in integrins. On the contrary, it inhibits adhesion by outcompeting the binding to other ligands, such as ICAM-1. It is highly expressed in the brain, restricts brain inflammation by inhibiting the entry of inflammatory cells (Choi et al., 2008). Additionally, Del-1 has been shown to have anti-inflammatory properties, reducing IL-17 production in leukocytes and relieving the symptoms of multiple sclerosis (Choi et al., 2015). It might be that ICAM-5 functions in similar ways to restrict microglia adhesion and cytokine production.

If ICAM-5 also regulates the expression and maturation of BDNF, it explains why ICAM-5 KO animals have an accelerated transition rate from filopodia to mature spine morphology. Although neurons are the main source of BDNF, microglia are known to secrete BDNF both in neuropathic pain and in non-pathological conditions (Coull et al., 2005; Parkhurst et al., 2013). We were not able to detect BDNF secretion from BV-2 cells or microglia, but this would be very interesting to investigate further. Since ICAM-5 is released from synapses where LTP is induced, it might well be that soluble ICAM-5 serves as a signal of active synapses to microglia. It would be tempting to suggest that cleaved ICAM-5 as a signalling molecule conveys the information from an active synapse to microglia, inducing BDNF secretion. We now found that in the absence of ICAM-5, BDNF levels are increased, indicating that in the normal brain the levels of BDNF could be suppressed by ICAM-5. This notion does not support our hypothesis, although the effect of ICAM-5 on BDNF could be implicated in early development, independently of microglia. ICAM-5 slows spine maturation (Matsuno et al., 2006) and BDNF could be another player here. These *in vivo* results need to be repeated with sibling matched WT and KO mice since maternal care and other external factors can influence the expression of BDNF. Moreover, it would be important to look at different developmental time points. Taken together, this suggests that ICAM-5 might slow spine maturation and prolong critical windows through yet a novel, BDNF related mechanism.

The expression of many proteins exhibits gender dimorphism and is spatially regulated. For instance, β2 integrins were recently shown to be upregulated in prenatal male mice, as compared to females and this was reversed right after birth. The same pattern was true for complement components (Prilutsky et al., 2017). As this study underlines, it is important to take the developmental stage and gender into consideration.

Since it has been difficult to address exactly how neurons tag the correct synapses for elimination, one could believe that additional mechanism might exist. The phage display provided clues that complement components might be bound to ICAM-5. If this is true, then the release of ICAM-5 from active synapses would be an elegant system for active synapses

to shed the complement tag and hence be spared from pruning. However, even this model does not explain the whole picture, since neurons lacking ICAM-5 still have an abnormal filopodia to spine maturation rate. In figure 14, two speculative mechanisms for how ICAM-5 regulates synaptic maturation are sketched.

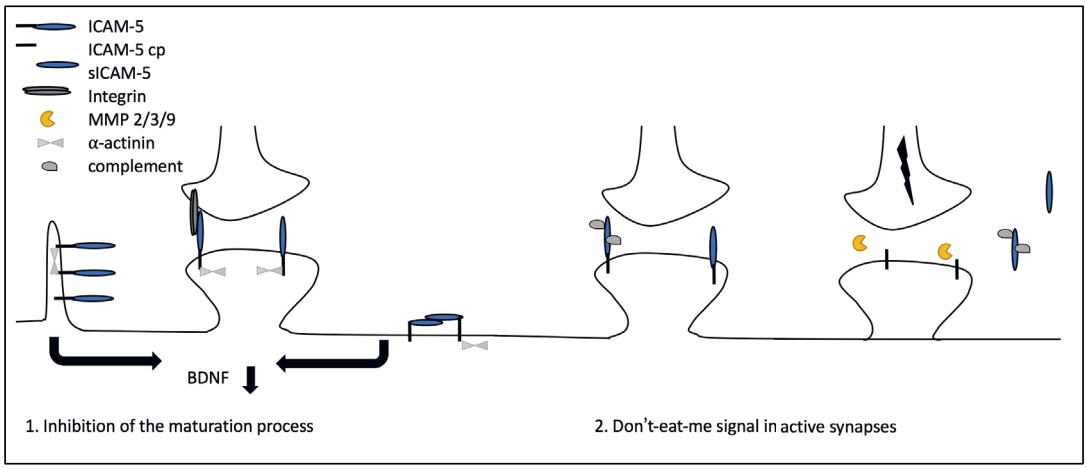

Figure 16. Hypothetical model of how ICAM-5 influences synaptic maturation and pruning through 1. BDNF and 2. Complement components. First, ICAM-5 retards spine maturation by downregulating BDNF production, hence prolonging critical windows. Second, postsynaptic elements are tagged by complement components, binding to ICAM-5. Active synapses are appropriately relieved of the tag as MMPs cleave ICAM-5.

Cytokines play a substantial role in neuronal function. Various cell types in the CNS secrete cytokines, and neurons can modulate the expression profile of microglia (Biber et al., 2007). Proinflammatory cytokines and LPS can inhibit the induction of LTP, while anti-inflammatory cytokines, such as IL-1ra, can diminish their action (Cunningham et al., 1996). The exact mechanism of how neurons exert this effect has not been completely understood. We have identified a modulatory role for ICAM-5 on both synaptic transmission and cytokine expression. Both isolated microglia and mixed glia in culture react to soluble ICAM-5, and in both cases, the effect was anti-inflammatory.

Isolated primary microglia treated with purified recombinant ICAM-5 D1-9 showed a clear anti-inflammatory effect measured by ELISA of secreted cytokines. To screen for a more general effect of endogenously secreted ICAM-5 on a mixed glial cell culture we used the cytokine array. These results are interesting, but should be considered as suggestive. A screen like this is based on a single sample and the exact cell population is not defined. Further, the conditioned culture medium could be considered a stress-response media, since it is nutrient deprived and supplemented with high levels of NMDA. However, the two

samples were treated exactly in the same way, with the sole difference that one neuron culture does not express ICAM-5. Hence, the differences observed should largely be dependent on ICAM-5, even though the pathway remains elusive.

Curiously, in our data set, IL-1ra was upregulated by ICAM-5. Fractalkine receptor knockout mice had hippocampal mediated learning disabilities and this effect was abolished by intra-hippocampal infusion of IL-1ra. Since IL-1ra is an antagonist for IL-1β, it was hypothesized to be the cause of the disability in these mice (Cunningham et al., 1996). It would be very interesting to investigate the role of ICAM-5 on IL-1ra in the future. Also, previous studies used a similar cytokine array to study the effect of ICAM-5 in herpes simplex virus type-1 infection (Tse et al., 2009). It was shown that ICAM-5 binds a particular gene product of the virus and in the study, they infected mice with the WT herpes virus, or a modified virus, lacking the ICAM-5 binding protein. Interestingly, they found the largest difference in cytokine expression in IL-1ra, CXCL10 and CCL2. In addition, CXCL11, CXCL9, CCL3, RANTES, TIMP-1, sICAM-1 and IFNγ were slightly upregulated when mice were infected with the wild type virus. This assay was not sensitive enough to detect differences in TNF-α expression, thus they used quantitative PCR to study it. It was found, that the TNF-α levels peaked several days earlier in mice infected with the wild type virus.

The most striking factor that was upregulated by ICAM-5 in the cytokine array, was angiopoietin-2. It has been regarded as a proinflammatory cytokine based on the recruitment of tumor associated macrophages in cancer. However, the effect of angipoietin-2 might be miss-interpreted, since it maintains an anti-inflammatory milieu in the solid tumor and promotes vascular sprouting. Hence, it is associated with a poor cancer prognosis, but we are still far from the full picture of the effects of this cytokine (Scholz et al., 2015). Interestingly, angiopoietin-2 deficient mice showed increased levels of TNF-α and IL-6 in the blood plasma when challenged by LPS, as compared to their WT littermates (Kurniati et al., 2013).

Taken together, it seems clear that ICAM-5 has a modulatory role on cytokine and chemokine expression in the brain. However, teasing out what is the effect of what is not easy, since many cytokines play in concert and regulate each other in a complex matrix. For example, fractalkine signalling can also induce a proinflammatory response in microglia or BV-2 cells through the p38MAPK/protein kinase C (PKC) pathway, resulting in elevated TNF-α and IL-1β expression in ischemic conditions (Liu et al., 2015).

Since ICAM-5 is found in the plasma of patients with acute encephalitis (Lindsberg et al., 2002) and drug resistant epilepsy (Yuan et al., 2017), it is likely that ICAM-5 modulate not only the response of immune cells in the brain parenchyma, but also in the periphery. The effect of ICAM-5 on microglia in various pathological conditions such as AD, Autism spectrum disorder or epilepsy remains unfortunately outside of the scope of this study. However, it remains highly interesting and we have opened some doors, inviting further research on this topic.

CONCLUDING REMARKS

Glutamate signalling in neurons induces MMP-mediated proteolysis of the adhesion molecule ICAM-5. The cytoplasmic tail dissociates from the cytoskeletal anchor α-actinin, possibly allowing GluN1 containing glutamate receptors to access α-actinin. As a consequence, synaptic maturation is promoted. The soluble fragment of ICAM-5 is on the other hand bound by microglia. Dynamic integrins mediate the binding and the full length ecto domain of ICAM-5 induces rapid clustering of microglia. ICAM-5 inhibits microglia phagocytosis and tilts the inflammatory profile of microglia towards a more neuro-protective. ICAM-5 continues to surprise and it is evident that there is more to be discovered about this interesting protein.

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